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Supplied free of charge: 250 reprints will be sent to the senior author about three weeks after publication of the article.

Contents

Cancer Treatment Reports
Vol. 62, No. 5
May 1978

Page

COMMENTARY

Empiric Antibiotic Therapy 673
Stephen C. Schimpff and Joseph Aisner
Baltimore Cancer Research Center, National Cancer Institute, Maryland

ARTICLES

Chemo-Immunotherapy for Unresectable Bronchogenic Carcinoma 681
G. P. Sarna, B. B. Lowitz, C. M. Haskell, F. J. Dorey, and M. J. Cline
University of California at Los Angeles Center for the Health Sciences

Phase II Trial of Ftorafur With Mitomycin C Versus Ftorafur With Methyl-CCNU in Untreated Colorectal Cancer 689
Thomas Buroker, Bonnie Wojtaszak, Aydin Dindogru, Michael DeMattia, Laurence Baker, Carol Groth, and V. K. Vaitkevicius
Wayne State University School of Medicine, Detroit, Michigan

Acute Renal Failure After *cis*-Dichlorodiammineplatinum(II) and Gentamicin-Cephalothin Therapies 693
Juan C. Gonzalez-Vitale, Daniel M. Hayes, Esteban Cvitkovic, and Stephen S. Sternberg
Memorial Sloan-Kettering Cancer Center, New York, New York

Intra-Arterial Radionuclide Infusion: A New Technique to Assess Chemotherapy Perfusion Patterns 699
W. D. Kaplan, C. J. D'Orsi, W. D. Ensminger, E. H. Smith, and D. C. Levin
The Sidney Farber Cancer Institute, Boston, Massachusetts

Chemotherapy of Transplantable Mouse Tumors With *cis*-Dichlorodiammineplatinum(II) Alone and in Combination With Sarcolysin 705
Michael A. Presnov, Alexandra L. Konovalova, Loodmila F. Romanova, Zoya P. Sofina, and Anastasia I. Stetsenko
Cancer Research Center, Academy of Medical Sciences USSR, Moscow

Effect of Actinomycin D and Neoarsphenamine on Tumor Control and Skin Tolerance in the Rat 713
John E. Moulder, Pallas Sun Lo, and James J. Fischer
Yale University School of Medicine, New Haven, Connecticut

Page

Antimetastatic Action of Some Triazene Derivatives Against the Lewis Lung Carcinoma in Mice 721
T. Giraldi, P. J. Houghton, D. M. Taylor, and C. Nisi
Institute of Cancer Research, Sutton, Surrey, England

Therapeutic Implications of Cell Kinetic Changes After Cyclophosphamide Treatment in "Spontaneous" and "Transplantable" Mammary Tumors 727
Paul G. Braunschweiger and Lewis M. Schiffer
Allegheny General Hospital, Pittsburgh, Pennsylvania

Effect of a Transplantable Leukemia on Hematopoietic Stem Cells in AKR Mice 737
Alexander Nakeff and Frederick A. Valeriote
Washington University School of Medicine, St. Louis, Missouri

Therapeutic Value of Mepitiostane in the Treatment of Advanced Breast Cancer 743
K. Inoue, K. Okazaki, T. Morimoto, M. Hayashi, S. Uyama, H. Sonoo, Y. Koshiba, T. Takihara, Y. Nomura, J. Yamagata, H. Kondo, K. Kanda, and K. Takenaka
Tokushima University School of Medicine, Japan

BCNU-Induced Perturbations in the Cell Cycle of 9L Rat Brain Tumor Cells 747
Kazuhiro Nomura, Takao Hoshino, Kathy Knebel, Dennis F. Deen, and Marvin Barker
University of California, San Francisco

Rate of DNA Synthesis as an Indication of Drug Toxicity and as a Guide for Scheduling Cancer Therapy 755
Glynn P. Wheeler and Jo Ann Alexander
Southern Research Institute, Birmingham, Alabama

Comparison of Adriamycin-Induced Nucleolar Segregation in Skeletal Muscle, Cardiac Muscle, and Liver Cells 771
Jerome Merski, Yerach Daskal, and Harris Busch
Baylor College of Medicine, Houston, Texas

Comparative Antitumor Activity of Actinomycin Analogs in Mice Bearing Ridgway Osteogenic Sarcoma or P388 Leukemia 779
William C. Rose, Mary W. Trader, W. Russell Laster, Jr, and Frank M. Schabel, Jr
Southern Research Institute, Birmingham, Alabama

Distribution and Antitumor Activity of Adriamycin Given in a High-Dose and a Repeated Low-Dose Schedule to Mice 791
Maria Adele Pacciarini, Brunella Barbieri, Tina Colombo, Massimo Broggini, Silvio Garattini, and Maria Grazia Donelli
Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy

Evaluation of VP-16-213 in Malignant Lymphoma and Melanoma 801
James W. Cecil, Joseph M. Quagliana, Charles A. Coltman, Muhyi Al-Sarraf, Tate Thigpen, and Carl W. Groppe, Jr
Southwest Oncology Group

Page

Phase II Clinical Trial With Vindesine for Remission Induction in Acute Leukemia, Blastic Crisis of Chronic Myeloid Leukemia, Lymphosarcoma, and Hodgkin's Disease: Absence of Cross-Resistance With Vincristine 805
G. Mathé, J. L. Misset, F. De Vassal, J. Gouveia, M. Hayat, D. Machover, D. Belpomme, J. L. Pico, L. Schwarzenberg, P. Ribaud, M. Musset, Cl. Jasmin, and L. De Luca
Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, and Département d'Hématologie de Institut Gustave-Roussy, Villejuif, France

Peptichemio in Advanced Neuroblastoma 811
B. De Bernardi, A. Comelli, C. Cozzutto, G. Lamedica, P. G. Mori, and L. Massimo
Giannina Gaslini Institute and Children's Hospital, Genoa, Italy

Peripheral Neuropathy as a Complication of *cis*-Dichlorodiammineplatinum(II) Treatment: A Case Report 819
Amos Kedar, Michael E. Cohen, and Arnold I. Freeman
Roswell Park Memorial Institute, Buffalo, New York

Inhibitory Effect of Hydroxynitrodihydrothymine on the Growth of Ehrlich Ascites Tumor Cells 823
I. Mustea, A. Bara, R. Comes, and I. D. Postescu
Oncological Institute, Cluj-Napoca, Romania

BRIEF REPORTS AND PRELIMINARY COMMUNICATIONS

Actinomycin D in Childhood Acute Lymphocytic Leukemia 829
Daniel M. Green, Stephen E. Sallan, and Awtar Krishan
Sidney Farber Cancer Institute, Boston, Massachusetts

Chemotherapy of Gynecologic Cancer With Nitrosoureas: A Randomized Trial of CCNU and Methyl-CCNU in Cancers of the Cervix, Corpus, Vagina, and Vulva 833
G. A. Omura, H. M. Shingleton, W. T. Creasman, J. A. Blessing, and R. C. Boronow
Gynecologic Oncology Group

Phase II Study of Triazinate and Pyrazofurin in Patients With Advanced Breast Cancer Previously Exposed to Cytotoxic Chemotherapy 837
W. C. Nichols, L. K. Kvols, J. N. Ingle, J. H. Edmonson, D. L. Ahmann, J. Rubin, and M. J. O'Connell
Mayo Clinic and Mayo Foundation, Rochester, Minnesota

1,2:5,6-Dianhydrogalactitol in Advanced Breast Cancer 841
B. Hoogstraten, R. O'Bryan, and S. Jones
Southwest Oncology Group

VP-16-213 Chemotherapy for Advanced Squamous Cell Carcinoma and Adenocarcinoma of the Lung 843
R. T. Eagan, J. N. Ingle, E. T. Creagan, S. Frytak, L. K. Kvols, J. Rubin, and R. T. McMahon
Mayo Clinic and Mayo Foundation, Rochester, Minnesota

Page

LETTERS

Tumor Growth Kinetics, Therapeutic Differentials, and Design of Treatment Schedules
An Alternate View 845
T. E. Wheldon and G. F. Brunton
West of Scotland Health Boards, Glasgow
Authors' Reply 846
Larry Norton and Richard Simon
National Cancer Institute, Bethesda, Maryland

Failure of Streptozotocin in Rare Hormonally Active Malignancies 847
Raymond B. Weiss
West Virginia University Hospital, Morgantown

Unpredictable Hematologic Toxicity of Weekly DDMP 849
Mogens Hansen, Marianne E. Lau, and Heine H. Hansen
Finsen Institute, Copenhagen, Denmark

A Response to the Guidelines for Training in Medical Oncology 850
Michael B. Van Scoy-Mosher
Cedars-Sinai Medical Center, Los Angeles,California

ANNOUNCEMENTS 851

Symposium on "New Drugs in Cancer Therapy"
Fourth Congress of the Medical Oncology Society
Fellowship in Medical Oncology
Seminars in Oncology at Roswell Park Memorial Institute
23rd Annual Clinical Conference on "Cancer of the Genitourinary Tract"
Monograph on Non-Hodgkin's Lymphoma
Conference on"Combined Modalities: Chemotherapy/Radiotherapy"
Fifth Annual Chicago Symposium on "Cancer Immunology: Experimental and Clinical"
Fall Meeting of The American Society for Pharmacology and Experimental Therapeutics and the Society of Toxicology

The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

COMMENTARY

Empiric Antibiotic Therapy[1]

Stephen C. Schimpff* and Joseph Aisner[2]

Prolonged absolute granulocytopenia substantially increases the risk of infection (1). The practice of promptly instituting empiric broad-spectrum, bactericidal antibiotics in combination has become accepted practice for the febrile granulocytopenic cancer patient with the result that bacteremia is seen in substantially fewer febrile episodes, septic shock has become uncommon, and infectious death has been markedly reduced (2–4). Despite these improvements, there remain many important questions to which answers are at best incomplete: What are the most appropriate combinations of antibiotics? Will the attainment of antimicrobial synergy be of clinical value? Can the choice of therapy be based upon knowledge of the organisms colonizing in the patient? What toxicities can be expected from the various combinations currently in use? Once an organism is isolated and antimicrobial susceptibility studies are available, how should therapy be adjusted to obtain the best efficacy to toxicity ratio? What should be done for the patient who has been placed on antibiotics for fever but no site of infection has been recognized and all cultures are negative? If a fungal infection is proven or suspected, is antifungal therapy of any ultimate value? Should antifungal therapy be utilized empirically and, if so, in what setting? Finally, when should granulocyte transfusions be instituted? The data available are briefly summarized below with suggestions for a stepwise approach to management of these difficult problems.

The majority of infections in the granulocytopenic cancer patient are caused by a limited number of organisms, principally *Staphylococcus aureus* and the enteric gram-negative bacilli such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (5). These are among the organisms which colonize in the patient at or near the sites most commonly infected: lungs, pharynx, perianum, and skin. Infection develops when mucosal or integumentary damage, obstructive phenomena, etc, occur in conjunction with granulocytopenia (2,3). Gram-negative bacilli are normally found in the lower alimentary tract whereas colonization in the oropharynx by these organisms develops in association with many disease processes even in the absence of antibiotic "pressure" (6). Colonization with yeasts such as *Candida albicans* and *Torulopsis glabrata*, however, appears to depend, at least in part, on bacterial suppression by antimicrobials. Thus, infection with these yeasts tends to follow periods of antibiotic administration (7).

Not all organisms colonizing in the granulocytopenic patient will cause infection, suggesting that despite the impaired host defenses, virulence, on a relative basis, between colonizing organisms is important to infection development. For example, although colonization with *P. aeruginosa* is tantamount to infection during granulocytopenia, colonization with *Citrobacter*, *Proteus*, or other species of *Pseudomonas* infrequently results in subsequent infection (5). Surveillance cultures, that is, routinely repeated cultures of nose, gingiva, and rectum, will indicate that the majority of infections are indeed caused by organisms which were already colonizing in the patient at or near the site where infection developed. Moreover, about one half of the infections will be found to be caused by organisms which were acquired and began colonizing in the patient only after hospital admission (5). Often, these organisms have more resistant antimicrobial susceptibility patterns.

Although a variety of antibiotic combinations will "cover" the spectrum of the commonly infecting organisms, the most frequently used regimens are combinations of two or three of the following types of antibiotics: carbenicillin (or ticarcillin), aminoglycosides (gentamicin, tobramycin, or amikacin), and cephalosporins (cephalothin or cefazolin). In vitro disc susceptibility assays usually indicate the spectrum of sensitivity shown in table 1.

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[1] Received Nov 28, 1977; revised Mar 6, 1978; accepted Mar 13, 1978.

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TABLE 1.—Spectrum of sensitivity (in vitro disc susceptibility assays)

	Antibiotic		
Organism	Carbenicillin	Amino-glycoside	Cephalosporin
Pseudomonas aeruginosa	+	+	−
Echerichia coli	+	+	+
Klebsiella pneumoniae	−	+	+
Proteus species	+	+	±
Staphylococcus aureus	±	+	+
Streptococcus faecalis	+	±	−
Bacteroides fragilis	+	−	−

Each of the various combinations of these agents has been tested in clinical trials of infection during granulocytopenia, and each has been found to be efficacious (2,8–25). Overall, it can be fairly stated that any of the two- or three-drug combinations have, in general, approximately equal degrees of overall efficacy with about 70% of documented infections responding to therapy. There are some differences, however, which are of importance for the individual patient. First, an aminoglycoside is inadequate as the only active agent for *P. aeruginosa* bacteremia (eg, in the cephalothin plus gentamicin combination) in the granulocytopenic patient (8,9,12,25,26). Second, the patient's response to therapy for gram-negative bacteremia would appear to be better if a synergistic combination of effective agents is utilized in a given patient (2,23,24,27–29). Third, although the frequency of staphylococcal infections would suggest the use of a penicillinase-resistant antibiotic in the initial regimen, the combination of an aminoglycoside plus a carbenicillin has been found adequate as initial therapy until culture results are available (13,25). Fourth, the combination of cephalothin with an aminoglycoside has been found to have substantial nephrotoxic potential, especially in older patients (17,18,25,30).

A recent prospective randomized trial (European Organization for Research on Treatment of Cancer [EORTC] Antimicrobial Therapy Project Group) was undertaken in an effort to determine whether any one of the two-drug combinations noted (carbenicillin plus cephalothin, carbenicillin plus gentamicin, or cephalothin plus gentamicin) was of greater overall efficacy or toxicity in these patients (table 2) (25). An analysis of the 625 patient trials from 17 institutions indicated that each combination was of approximately equal general efficacy. There was a suggestion that the combinations with carbenicillin were superior for *P. aeruginosa* infection. The three combinations were equivalent for *K. pneumoniae* and *S. aureus*. The carbenicillin plus cephalothin regimen was least satisfactory for *E. coli* as a result of some isolates which were doubly resistant. As a result, the carbenicillin plus gentamicin combination had, marginally, the best overall efficacy as an initial empiric regimen against the four most common infecting organisms.

TABLE 2.—Effectiveness of 2-drug combinations (EORTC study)

	Response rate (No. of responses/No. treated)		
	Carbenicillin + cephalothin	Carbenicillin + gentamicin	Cephalothin + gentamicin
Pseudomonas aeruginosa	4/8	10/16	5/13
Klebsiella species	9/15	12/22	6/17
Escherichia coli	8/18	22/30	18/27
Staphylococcus aureus	18/22	12/13	13/19

Ticarcillin was substituted for carbenicillin in approximately one third of the patients in this trial. Efficacy was equivalent but dose-related side effects of hypokalemia and circulatory overload were often more readily controlled in the patients treated with ticarcillin (25).

Although most bacterial infections responded well, gram-negative bacteremias were the exception with a response rate of 55% for single pathogen bacteremias and 18% for multiple pathogen bacteremias. The primary determinant of response for gram-negative bacteremia, provided the patient received at least one active agent as judged by in vitro disc susceptibility, was whether or not there was at least a minimal return of the peripheral granulocyte count within about 1 week after onset of infection. Specifically, 88% of the patients with gram-negative bacteremia improved with such a rise in granulocyte count whereas only 22% improved when the granulocyte count remained stable or declined further. Thus, although prompt therapy often prolonged life, antibiotics alone generally could not cure the most serious infections without some improvement in host defenses (25). This has obvious potential implications for the use of granulocyte transfusions.

This trial was designed to determine if combinations with two active agents were more effective than combinations which included only one active drug for a given pathogen. Synergism between penicillin and streptomycin had long been known to be useful for treating *Streptococcus faecalis* endocarditis and by analogy it was presumed that synergistic combinations would be useful for the severe infections occurring in the compromised host. Previous trials in non-neutropenic cancer patients suggested the value of synergism for gram-negative infections, and the good results observed with carbenicillin and gentamicin for *P. aeruginosa* bacteremia in granulocytopenic patients encouraged the concept (12,14,27–29). Although > 100 bacteremias were evaluated in the EORTC trial, major differences were not found among the three regimens for the more commonly infecting organisms; rather, the major determinant of response for bacteremias was the direction of granulocyte count change (25). Animal studies, however, have demonstrated the value of utilizing synergistic combinations and a recent clinical study at the University of California at Los Angeles provided further support for this approach (24,31). In the latter trial, among 40 patients with gram-negative bacteremia, 18% died if the regimen utilized was synergistic to the infecting pathogen, whereas 40% died if the two drugs were both active but not synergistic and 71% died if only one agent was active (24). A recent paper in this journal by Klastersky et al noted that the serum bactericidal level was higher in granulocytopenic patients who received an antibiotic combination which showed in vitro synergy; therapeutic responses correlated with the serum bactericidal value (23). In conjunction with the EORTC trial, we have studied the bactericidal activity of patients' serum 1 hour after antibiotic administration against multiple strains of the four most common pathogens (32). Sera from patients receiving any of the three regimens showed substantial bactericidal activity against *S. aureus* (geometric mean titer, $> 1{:}28$) and *E. coli* ($> 1{:}12$). Against *P. aeruginosa*, both ticarcillin-containing regimens showed some although minimal bactericidal activity (geometric mean titer, 1:2 and 1:2), whereas the cephalothin plus gentamicin regimen had, on the average, no activity. Conversely, the cephalothin plus gentamicin regimen had the best serum bactericidal activity against *K. pneumoniae* (1:34), although the other regimens did have measurable effects (ticarcillin plus cephalothin, 1:6, and ticarcillin plus gentamicin, 1:17). Considered together, these would suggest an advantage for synergistic antimicrobial combinations.

It can be argued, however, that the poor results attributed to aminoglycosides acting alone have been related to low blood concentrations and not to an intrinsic ineffectiveness of the antibiotic (26). At our institution, samples taken 1 hour after iv administration of gentamicin averaged 2.8 μg/ml in the cephalothin plus gentamicin regimen and 2.0 μg/ml in the ticarcillin plus gentamicin regimen (32). In this regard, investigators at the M. D. Anderson Hospital and Tumor Institute, Houston, Tex (33,34), have recently suggested that the efficacy of aminoglycosides is substantially improved when they are given by continuous infusion. With improved serum levels, they find that the aminoglycoside alone is as efficacious as the aminoglycoside combined with carbenicillin, provided that there is some rise in the granulocyte count during therapy (34).

Of equal importance to efficacy is the possibility for antibiotic-induced toxicity, especially the potential for serious renal damage secondary to regimens employing an aminoglycoside. It had been suggested that the combination of cephalothin plus an aminoglycoside was more nephrotoxic than either agent utilized alone (35,36). In the multi-institutional EORTC trial, which utilized antibiotic doses generally considered maximal, there was significantly more antibiotic-associated severe nephrotoxicity (defined as a rise in creatinine from normal to > 2.5 mg/100 ml) among the patients who received cephalothin plus gentamicin (12%) than in those who received either carbenicillin plus gentamicin (2%) or carbenicillin plus cephalothin (4%). Moreover, it was noted that nephrotoxicity occurred primarily in the older patient population with a high normal serum creatinine; at > 60 years of age there was a 27% incidence of severe nephrotoxicity among those receiving cephalothin plus gentamicin (25). Although animal data are not in accord with these findings (37,38), two other prospective randomly controlled trials confirm that cephalothin plus an aminoglycoside (gentamicin or tobramycin) is more nephrotoxic than the use of the aminoglycoside with either carbenicillin, ticarcillin, or methicillin (18,30). Not all clinical trials in which these two types of antibiotics have been used together support these observations (16,20,22,27); nevertheless, it currently seems prudent to avoid the use of the two-drug combination of a cephalosporin and an aminoglycoside, especially among older patients with borderline renal function unless dictated by *Klebsiella* septicemia (32,39,40). The nephrotoxic potential of the three-drug combination (carbenicillin, aminoglycoside, and cephalosporin) would also appear to be substantial based on some but not all trials of that regimen used alone or in comparison with cephalothin and carbenicillin (15,17,23). Finally, a report appearing in this current issue noted four patients who developed severe renal failure when cephalothin and gentamicin were administered shortly after the use of *cis*-dichlorodiammineplatinum(II) (41).

A satisfactory current choice for initial empiric antibiotic therapy at many institutions would be the combination of ticarcillin plus gentamicin. It would be appropriate to use a different aminoglycoside such as tobramycin when *P. aeruginosa* is highly suspected (because of the slightly greater activity against this organism) or amikacin in a hospital setting where the frequency of gentamicin-resistant gram-negative bacilli is substantial (because fewer organisms are resistant to amikacin than to gentamicin or tobramycin). Since it has been shown that most infections are caused by the organisms already colonizing in these patients, a reasonable aid to antibiotic choice is to obtain serial "surveillance cultures" on high-risk patients such as those with acute leukemia receiving induction chemotherapy (5). From these cultures (particularly gingiva and rectum) it can be determined which potential pathogens are present and therefore which are the most likely to cause infection. Additionally, results of susceptibility tests can help to establish a rational choice of drugs including the appropriate aminoglycoside. The use of continuous surveillance cultures is expensive, however, so that a practical alternative is to design empiric antibiotic therapy based on known patterns of infection within the individual hospital and unit and upon the patterns of antimicrobial susceptibility encountered in recent months in that unit. This approach requires constant update and review of infection data.

Since the possibility for bacteremia and bacteremic shock is substantial if the patient is not treated promptly, it is appropriate to treat the granulocytopenic patient when fever first develops unless there is reasonable evidence that the fever is caused by a noninfectious process. However, there is no unified opinion as to when to discontinue antibiotics when no definite evidence of infection has been found after 3–4 days. On the one hand, it has been suggested that antibiotics should be continued if the patient remains febrile in the absence of a defined infection because stopping antibiotics may allow a subclinical infection the opportunity to escape control and overwhelm the patient before therapy can be reinstituted. An alternate approach used successfully at our Center is to discontinue antibiotics after approximately 4 days if a *complete* and *thorough* repeat history, examination, and laboratory evaluation are all nonrevealing, if the patient is febrile yet appears stable and nontoxic, and if all original culture data are nonrevealing (2). This approach has the advantage of withdrawing the antibiotic pressure that is likely to encourage either resistant bacteria or fungi from causing infection (25), but for any given patient the decision should be based upon very careful repeated evaluation and continued close followup so that therapy can be promptly reinstituted if warranted. If, on the other hand, no infection is documented yet the patient has had an obvious response to antimicrobial therapy ("possible infection"), it seems reasonable to continue the original antibiotic regimen for approximately 5 more days or a total of about 7–10 days of therapy (42).

Having once instituted empiric antibiotic therapy, a decision must be made with regard to granulocyte transfusions. Most infections in granulocytopenic patients including gram-negative bacteremias will respond to the appropriate use of prompt empiric antibiotic therapy alone; therefore, not all granulocytopenic patients need receive wbc transfusions. By reviewing recent antibiotic trials, reasonable criteria can be developed to guide the institution of granulocyte transfusions. The patients who are less likely to respond are those with a gram-negative bacteremia, pneumonia, or both in the setting of an aplastic bone marrow with persistent granulocytopenia (2,8,10,13,19,25). In recent studies of granulocyte transfusions, patients who exhibited early spontaneous bone marrow recovery had a substantially improved prognosis with gram-negative bacteremia. Persistence of an aplastic marrow has not usually been associated with complete infection resolution with antibiotic therapy alone (43–46). It therefore seems appropriate to institute granulocyte transfusions in most patients with known or highly suspected gram-negative bacteremia and an aplastic marrow which is not expected to return to normal function in the next 7–14 days. Likewise, for the persistently granulocytopenic patient with a pneumonia or other serious systemic infection which is not clinically improving after 24–48 hours of appropriate antibiotic therapy, the addition of granulocyte transfusions is indicated (47,48). As a general rule, this should mean that ≤ 10% of granulocytopenic patients receiving empiric antibiotic therapy should need the addition of granulocyte transfusions. When granulocyte transfusions are indicated, however, there is no question that their prompt application will be of substantially greater benefit than if the clinician delays their use until the patient's condition is critical (48). In this regard, potential donors for patients at high risk of infection at this Center are identified in advance and notified at the first evidence of infection so that granulocyte transfusions can be begun within 24–48 hours if indicated by the above criteria (49).

As parenteral broad-spectrum antibacterial agents receive more use and as granulocytopenic patients survive longer, an increased number of fungal infections are being encountered (7,50,51). The observation of an increased occurrence of fungal infections, such as *Aspergillus* spp, *Candida* spp, *T. glabrata*, and others, raises a number of questions relating to the efficacy of the currently available agents, the prospects for new agents or combinations of agents, and the indications for empiric institution of antifungal therapy.

In the past there has been a relatively pessimistic approach to the treatment of systemic mycoses in the myelosuppressed patient and, in general, the outcome of treatment of the systemic mycoses has been dismal (50–53). An examination of the clinical course of such patients, however, indicates that the diagnosis of disseminated fungal infection has generally been made many days to weeks after the onset of infection because of the hesitancy to do the invasive procedures necessary to establish the proper diagnosis (51). It is not surprising that the resultant late institution of therapy has been relatively ineffective, especially in such diseases as aspergillosis where the natural history of the disease produces arterial invasion, distal infarction, and then invasion of the necrotic tissue by the fungus in a site where antibiotics probably permeate poorly. There have been several recent reports of the successful treatment of invasive aspergillosis, and at this Center, success has been associated with earlier diagnosis (table 3) (54–56). Thus, an aggressive diagnostic approach is likely to produce better therapeutic results with fungal infections provided amphotericin B is begun promptly (0.3 mg/kg on Day 1 and 0.6 mg/kg/day thereafter).

Sputum samples from neutropenic patients are generally useless for the evaluation of pulmonary infiltrates because of contamination with oropharyngeal flora (57). If coagulation parameters permit, the first step should be transtracheal aspirate followed, if negative, by more invasive techniques such as transtracheal bronchial brush biopsy, fiberoptic bronchoscopy, or open or closed lung biopsy (57–59). Utilizing this type of stepwise approach, our experience for the past several years has been that the etiologic agent can be established in about 50% of patients so that appropriate therapy can be promptly instituted with discontinuation of unneeded, potentially toxic agents.

The only drugs currently available include amphotericin B and 5-fluorocytosine as well as the experimental agent miconazole (51,60). The latter agent has not been satisfactorily established as clinically effective for invasive candidiasis or aspergillosis, especially in the granulocytopenic patient, thus leaving the current choice of treatment essentially to the two agents, amphotericin B and 5-fluorocytosine. Although 5-fluorocytosine has been reported to have activity in the treatment of patients with several systemic mycoses, especially *Candida* or *T. glabrata* infections, its efficacy in the neutropenic patient has not been adequately established and resistance in many species has been noted (61). Amphotericin B has been shown to be effective in a variety of systemic mycoses; however, this agent has significant morbidity including frequent induction of renal impairment (60). With the only known active drug having such substantial toxicity, there is little enthusiasm for using it as part of early empiric therapy. Furthermore, fungal infections are uncommon relative to bacterial infections so that the use of empiric amphotericin B is unwarranted except in a setting where many of the epidemiologic factors associated with systemic mycoses are present (25). Most granulocytopenic patients who develop fungal infections do so during or following several prolonged courses of antibacterial therapy. The yeasts such as *Candida* and *T. glabrata* are often acquired during hospitalization, with infection developing after the organism has been found to be colonizing in multiple body sites (7,62,63). The filamentous fungi are also often acquired in the hospital (64). For *Aspergillus flavus* there has been an association noted at this Center between antibiotic suppression of nasal bacteria, subsequent local colonization, and infection with *A. flavus* followed, if the patient remains granulocytopenic, by local or systemic invasive infection (56). Thus, any patient who develops a new febrile episode while granulocytopenic and who has received prolonged broad-spectrum antibiotic therapy should be considered for possible empiric antifungal therapy. The decision, prior to results from potentially invasive procedures, can be based on epidemiologic factors such as those suggested above. In patients who cannot tolerate invasive procedures because, for example, of uncorrectable bleeding parameters, the choice of therapy may rest primarily upon consideration of the predisposing factors present.

Amphotericin B is not only toxic but requires prolonged administration, often over months, with slow resolution of many infections (60). Another approach is to use combinations of antifungal agents which might be synergistic or to use antifungal agents with other compounds which might augment activity. Many studies have utilized the combination of amphotericin B and 5-fluorocytosine, and the combination has been shown to be superior for *Cryptococcus neoformans* infection (61). Recent

TABLE 3.—Response of invasive *Aspergillus* infection to amphotericin B therapy (ref 56)

Time from onset of infection to start of therapy	No. of—	
	Patients	Responses
1–4 days	6	6*
≥ 14 days	6	0
≥ 14 days†	5	—

*3 complete responses; 3 partial responses.
†Diagnosis only at necropsy; amphotericin B never given.

in vitro studies have shown that the combination of amphotericin B and rifampin are synergistic for a variety of filamentous and yeast-like fungi including *Candida* and *Aspergillus* (65–67). Animal studies have indicated superiority for this combination; however, clinical data are not yet available as to whether this combination is more effective than amphotericin B alone. Another potential, but clinically untested, approach is to utilize solubilizing agents such as dimethylsulfoxide, which might increase the tissue penetration of agents such as amphotericin B into relatively avascular sites (eg, aspergillosis in a sinus cavity). Finally, since these infections tend to occur principally in the most severely granulocytopenic patients, it would seem logical to include granulocyte transfusions in the therapeutic program. There have been several reports of granulocyte transfusions, given in adjunct to antifungal therapy, claiming a beneficial role (68–70), but at present there is little information to determine if they are of any substantial benefit to patients with disseminated mycoses.

In summary, clinical experience indicates that the prompt use of antibiotic therapy in an empiric manner in the febrile, granulocytopenic patient is appropriate. Although most antibiotic combinations will prove to have approximately equal degrees of efficacy in an overall sense, the more severe infections will probably be benefitted by the use of combinations synergistic for the infecting organism. For most patients a combination of ticarcillin plus gentamicin will prove to be appropriate empiric therapy, but variations to this regimen can and should be made based on both institutional and individual patient considerations. Cephalothin plus an aminoglycoside should be avoided when possible because of the combined nephrotoxic potential. Consideration should be given to discontinuing empiric antibiotic therapy after 3–5 days for those patients in whom fever was not apparently secondary to infection. Granulocyte transfusions are indicated for the granulocytopenic patient who either is not responding adequately despite appropriate antimicrobial drugs or who is unlikely to respond because of the combination of a serious systemic infection plus persistent, marked granulocytopenia. Finally, aggressive measures should be utilized when indicated to diagnose fungal infections followed by immediate institution of full doses of amphotericin B, since many fungal infections will respond if treated promptly. The empiric use of amphotericin B should be reserved for those situations in which epidemiologic factors strongly suggest the possibility of fungal infection but where documentation is not feasible.

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Chemo-Immunotherapy for Unresectable Bronchogenic Carcinoma[1,2]

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SUMMARY

Seventy-nine patients with metastatic or unresectable bronchogenic carcinoma were treated with a regimen of combination chemotherapy which included methotrexate, Oncovin (vincristine), cyclophosphamide, and adriamycin (MOCA), and were randomized to receive no additional therapy, immunotherapy with bacillus Calmette-Guérin, or immunotherapy with *Corynebacterium parvum* (*C. parvum*). The response rate and estimated median survival time were 68% and 42 weeks in small cell carcinoma and 18% and 29 weeks in other histologic types. Improved survival correlated with high performance status and response to therapy. Immunotherapy did not improve response, time to progression, or hematopoietic tolerance of chemotherapy. *C. parvum* was associated with significant morbidity and was poorly tolerated. MOCA appears to be of modest value in the treatment of bronchogenic carcinoma, particularly of the small cell type. A role for immunotherapy remains unproven.

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Although single-agent chemotherapy is relatively ineffective treatment for unresectable, recurrent, or metastatic ("advanced") bronchogenic carcinoma (1), there are data suggesting that combination chemotherapy and immunotherapy may each be useful. Combination chemotherapy has been associated with improved response rates and survival for small cell carcinoma (2,3) as well as for other histologic types (4,5) of lung cancer. Immunotherapy has also been said to show promise in the treatment of bronchogenic carcinoma, both for resected (6) and for unresectable (7,8) disease.

We have treated 79 patients with advanced bronchogenic carcinoma with a program of multiple-agent therapy alone or in combination with immunotherapy. Our objective was a randomized controlled study to determine the effects, if any, of immunotherapy with *Corynebacterium parvum* (*C. parvum*) or bacillus Calmette-Guérin (BCG) upon the response rate, survival, and hematologic tolerance of chemotherapy in patients treated with the combination-chemotherapy regimen of methotrexate, Oncovin (vincristine), cyclophosphamide, and adriamycin (MOCA). We also sought to determine the effects of this chemotherapy upon response rate and survival.

PATIENTS AND METHODS

Patients with unresectable, metastatic, or recurrent bronchogenic carcinoma were considered eligible for this protocol with the following exclusions: age > 70 years; performance status (9) < 50%; brain metastases; previous chemotherapy; previous radiation therapy involving > 25% of bone marrow-bearing sites; or cardiac disease precluding the use of adriamycin. Patients who had received past radiation therapy were eligible only if active disease was

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TABLE 1.—Dose levels for MOCA

	Drug, dose
Standard dose, administered iv q2wks	Methotrexate, 50 mg/m^2 Vincristine, 1.4 mg/m^2 (≤ 2 mg total dose) Cyclophosphamide, 500 mg/m^2 Adriamycin, 30 mg/m^2
Wbc count 3000–4000/mm^3 and/or platelet count 100,000–125,000/mm^3	50% of standard-dose methotrexate, cyclophosphamide, and adriamycin; full dose of vincristine
Wbc count 2000–2999/mm^3 and/or platelet count 75,000–99,999/mm^3	25% of standard-dose methotrexate, cyclophosphamide, and adriamycin; full dose of vincristine
Wbc count ≤ 2000/mm^3 and/or platelet count < 75,000/mm^3	No chemotherapy

present. No patient with limited disease received primary radiation therapy. Two patients with extensive disease received simultaneous local radiation for superior vena cava obstruction (BCG) and bronchial obstruction (no immunotherapy). Disease was staged with chest roentgenogram ± tomography, multiple bone roentgenograms, radionuclide scans of brain, bone, liver, and spleen, and bone marrow aspirate and biopsy. Disease limited to one hemithorax plus ipsilateral supraclavicular nodes was considered limited. All other disease was considered extensive. Repetitive studies included a physical examination and a cbc count at each visit and monthly screening chemistries (SMA-12) and chest roentgenograms. Abnormal scans were repeated every 6 weeks and abnormal bone marrows every 12 weeks.

All patients received biweekly chemotherapy with MOCA after premedication with prochlorperazine at a dose of 10 mg im. A biweekly schedule was chosen to test the ability of *C. parvum* or BCG to enhance hematopoietic recovery and to minimize the interval in which tumor cells might recover from chemotherapy. Standard dose levels as well as dosage adjustments for hematologic toxicity are listed in table 1. Adriamycin total dose was limited to 550 mg/m^2 or to 450 mg/m^2 in patients with previous mediastinal irradiation. Initially, patients were randomized to receive immunotherapy with *C. parvum*, immunotherapy with BCG, or no immunotherapy. *C. parvum* (Burroughs Wellcome Co, Research Triangle Park, NC) was administered iv over 1 hour at a dose of 2.5 mg/m^2 after premedication with acetaminophen at a dose of 15 g orally and prochlorperazine at a dose of 10 mg im. BCG (Chicago Research strain) was applied to the skin in four quadrants on the thorax and abdomen by the tine technique at a dose of 10^8 live organisms at each site. The *C. parvum* arm was discontinued after 19 patient entries because of undue toxicity (*vide infra*). Immunotherapy was given weekly for 12 weeks and then biweekly. Detailed immunologic assessments were performed prior to and during therapy, but those data are not the subject of this report.

Characteristics of each treatment group are detailed in table 2. The groups were comparable as to age, sex, and tumor histology. The BCG group, however, was weighted toward patients with distant tumor and low performance status, while the *C. parvum* group had received less radiation therapy.

Complete response (CR) was defined as the disappearance of all measurable disease. Partial response (PR) was defined as a 50% decrease in the sum of the products of the greater and lesser diameters of measurable lesions without the appearance of new lesions. Minimal response (MR) was defined as a decrease in the size of measurable lesions not meeting the 50% criterion. Progression was defined as the appearance of new lesions or an increase in the size of old lesions. Five patients had disease not readily evaluable (eg, carinal tumor seen at bronchoscopy or mediastinal nodes seen at mediastinoscopy); two patients were clinically stable but had not received re-evaluation of marker areas at the time of analysis. These patients were judged inevaluable for the determination of response rate, but were included in the evaluation of survival.

Survival curves are plotted in an actuarial fashion by the method of Kaplan and Meier (10). Tests for significant differences between survival curves were performed by the Cox (11) and Breslow (12)

TABLE 2.—Characteristics of treatment groups (all patients received chemotherapy)

Immunotherapy	No. of patients	Median age	Small cell histology	Distant metastases	Mean performance status	Past or present radiation therapy
C. parvum	19	54	42%	79%	87%	11%
BCG	31	57	39%	74%	72%	23%
None	29	56	31%	66%	78%	24%
Totals	79	56	36%	72%	78%	20%

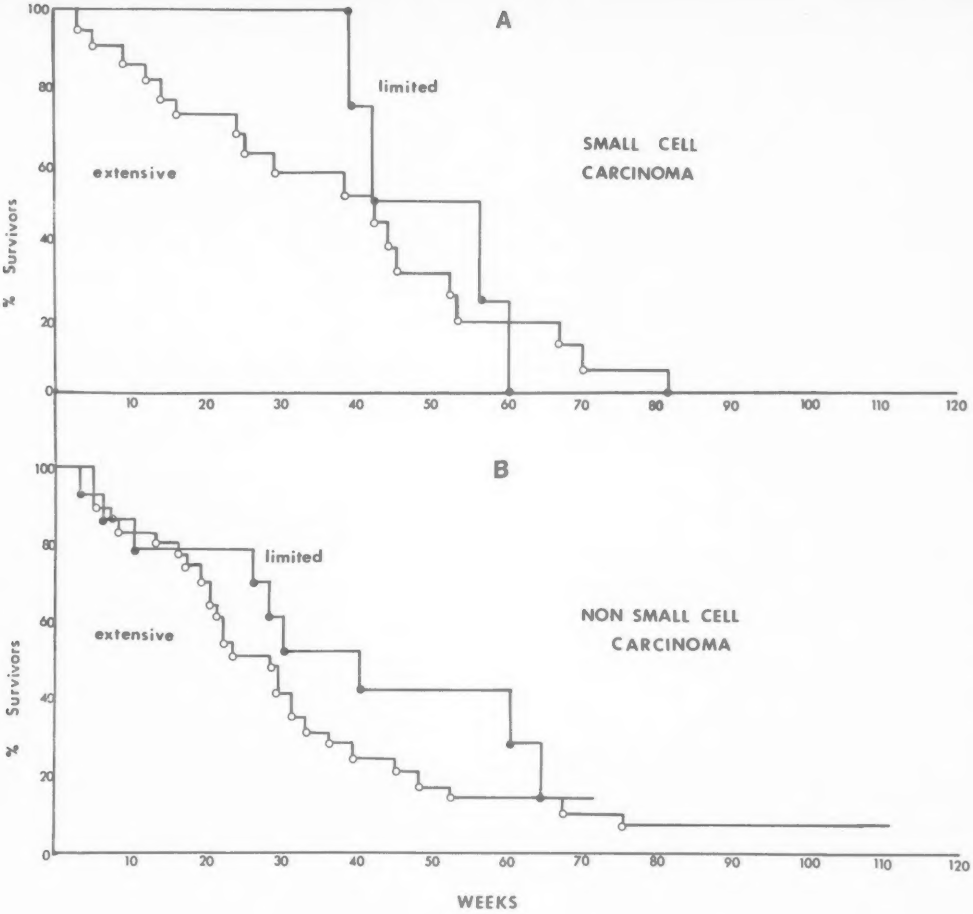

FIGURE 1.—A. Kaplan-Meier (10) plot of estimated survival for patients with limited small cell carcinoma (4 deaths, 3 patients censored) and extensive small cell carcinoma (18 deaths, 4 patients censored). B. Kaplan-Meier (10) plot of estimated survival for patients with limited non-small cell carcinoma (9 deaths, 6 patients censored) and extensive non-small cell carcinoma (29 deaths, 6 patients censored).

techniques. Statistical analysis of other data was by the chi-square test.

RESULTS

Response Rate

The CR plus PR rate for small cell carcinoma of the lung was 68% (29% CR rate) and for other histologies was 18% (5% CR rate). Within those other histologies, the response rate was 14% in epidermoid carcinoma, 15% in adenocarcinoma, and 30% in large cell carcinoma.

Response rates were highest in the group without immunotherapy for both small cell carcinoma (78% versus 67% for BCG and 57% for *C. parvum*) and non-small cell carcinoma (25% versus 21% for BCG and 0 for *C. parvum*). Those differences in response rate with respect to immunotherapy were not significant at the $P = 0.05$ level. An additional four patients with non-small cell carcinomas (9%) had MRs.

Survival

Nineteen of 79 patients (24%) remained alive at the time of analysis with a median followup of 30 weeks. Estimated median survival is 42 weeks for patients with either local or extensive small cell carcinoma and 29 weeks for patients with other histologies (40 weeks for local disease, 28 weeks for extensive disease) (fig 1). Improved survival was

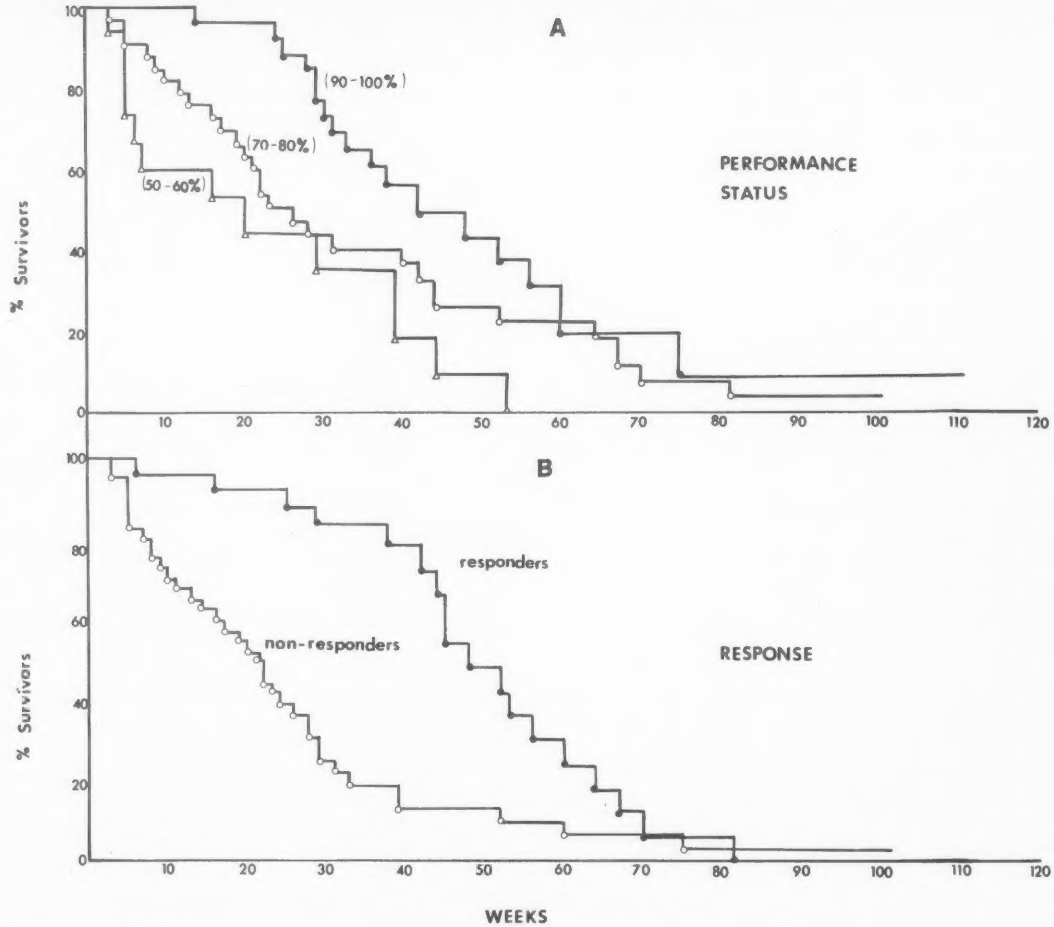

FIGURE 2.—A. Kaplan-Meier (10) plot of estimated survival for patients with an initial performance status of 50%–60% (13 deaths, 3 patients censored), 70%–80% (29 deaths, 6 patients censored), and 90%–100% (18 deaths, 10 patients censored). B. Kaplan-Meier (10) plot of estimated survival for responders (CR + PR; 18 deaths, 9 patients censored) and nonresponders (stable disease or progression; 36 deaths, 5 patients censored). 4 patients with MR had a median survival of 52 wks. 7 patients were unevaluable.

associated with high performance status ($P < 0.01$) (fig 2) and CR or PR to therapy ($P < 0.01$) (fig 2). The four patients with non-small cell disease and MRs had a median survival of 52 versus 21 weeks in all nonresponders and 22 weeks in nonresponders with histologies other than small cell.

No improvement of survival is seen when all patients treated with BCG or *C. parvum* are compared to all patients not receiving immunotherapy (fig 3). For patients with small cell carcinomas, however, the median survival was greatest in the *C. parvum* group (36 weeks versus 26 weeks for BCG and 22 weeks for no immunotherapy), while in the other histologic types, the median survival was least in the *C. parvum* group (25 weeks versus 44 weeks for BCG and 53 weeks for no immunotherapy) (fig 3). These differences with respect to immunotherapy are not significant at the $P = 0.05$ level. Time to progression did not vary with immunotherapy (medians of 21, 23, and 21 weeks for *C. parvum*, BCG, and no immunotherapy respectively).

Toxicity and Hematologic Tolerance to Chemotherapy

Administration of MOCA was associated with frequent nausea and anorexia and with occasional vomiting. Alopecia was universal and paresthesias frequent. Mucositis was infrequent but occasionally

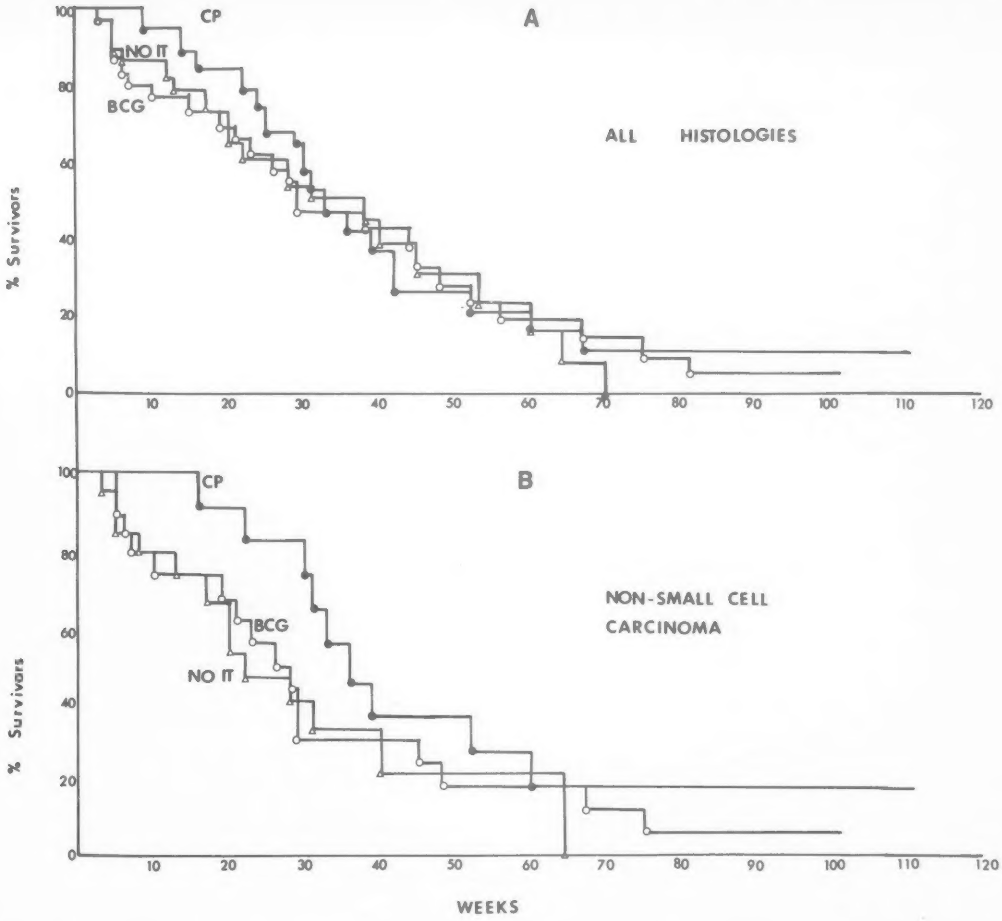

FIGURE 3.—A. Kaplan-Meier (10) plot of estimated survival for patients with all histologies of carcinoma of the lung receiving *C. parvum* (17 deaths, 2 patients censored), BCG (24 deaths, 7 patients censored), and no immunotherapy (19 deaths, 10 patients censored). B. Kaplan-Meier (10) plot of estimated survival for patients with non-small cell carcinoma of the lung receiving *C. parvum* (9 deaths, 2 patients censored), BCG (16 deaths, 3 patients censored), and no immunotherapy (13 deaths, 7 patients censored).

severe. One episode each of cystitis and congestive heart failure was observed. There were 11 episodes of serious infection following 617 courses of MOCA (risk, 2% per dose and 13% per patient). Individual patients received from one to 30 courses (median, six) of MOCA. No fatalities were attributed to complications of therapy.

C. parvum was poorly tolerated. Chills and fever were universal; two patients had moderate hypotension. One patient had diplopia and another had toxic epidermal necrolysis associated with but not necessarily caused by *C. parvum.* Ten of 19 patients found *C. parvum* unacceptable and refused further immunotherapy after one to 12 doses (median, four). The *C. parvum* randomization was accordingly discontinued.

BCG was associated with local skin reactions and rare fever. Acid fast bacilli were seen in the urine of one patient, but *Mycobacterium* cultures were negative. Another developed a granulomatous skin lesion with negative cultures and acid fast stains.

Cumulative dosage of administered cytotoxic chemotherapy (methotrexate, cyclophosphamide, and adriamycin) was 70% of the projected standard dose. There was no difference in tolerance of chemotherapy among the *C. parvum*, BCG, and no immunotherapy groups. Table 3 presents the incidence of severe leukopenia (< 1500 cells/mm^3), granulocy-

TABLE 3.—Incidence of hematopoietic toxicity 2 wks after full-dose MOCA

Immuno-therapy	Wbc count $<1500/\text{mm}^3$	Polymorpho-nuclear neutrophil count $\le 750/\text{mm}^3$	Platelet count $\le 50{,}000/\text{mm}^3$
C. parvum	0	55%	9%
BCG	6%	20%	6%
None	2%	25%	1%
Totals	3%	28%	4%

topenia ($\le$ 750 cells/mm^3), and thrombocytopenia ($\le$ 50,000 cells/mm^3) 2 weeks after standard doses of MOCA for each immunotherapy arm. *C. parvum* was associated with more frequent episodes of severe granulocytopenia (*C. parvum* versus no immunotherapy, $P < 0.02$; *C. parvum* versus BCG, $P < 0.01$). Differences in leukopenia and thrombocytopenia were not statistically significant at the $P = 0.05$ level.

DISCUSSION

The rationale for trials of immunotherapy in bronchogenic carcinoma has been reviewed by Hersh et al (13,14) who have cited evidence for the existence of lung tumor antigens as well as for the frequency of impaired cellular immunity and its correlation with prognosis in patients with bronchogenic carcinoma. Adjuvant immunotherapy for resected bronchogenic carcinoma with intrapleural BCG (6) and with oral levamisole (7) has shown promise, as has BCG when added to "radical" radiation therapy for unresectable local bronchogenic carcinoma (15). *C. parvum*, given sc or iv along with combination chemotherapy, has been reported to prolong survival in patients with a variety of malignancies, including bronchogenic carcinoma (8,16,17). This prolongation of survival has been attributed to an improved duration of positive response and of stability rather than to an improved response rate. *C. parvum* has also been reported to improve hematopoietic tolerance of chemotherapy, and thus to increase delivered doses of chemotherapy (16,17). Iv *C. parvum* has been reported to result in a 40% PR rate in patients with unspecified malignancies (16). It has also been postulated that the iv route for administration of *C. parvum* may be effective in dealing with tumors in the lung or liver (8,18).

In contrast to these reports, our study did not indicate that immunotherapy with *C. parvum* or BCG conferred clear benefit to patients with lung cancer. At best, modest improvement of survival was seen with *C. parvum* in non-small cell histologies. No such improvement was seen with immunotherapy in small cell disease. The failure of immunotherapy to prolong survival in small cell carcinoma has also been reported with the methanol extracted residue of BCG (19) and BCG (20). Additionally, regardless of histology, neither BCG nor *C. parvum* was associated with improved response rate or time to progression in our patients.

C. parvum was not shown to improve hematopoietic tolerance to chemotherapy or to permit administration of increased doses of chemotherapy. On the contrary, administration of *C. parvum* was associated with a trend toward decreased granulocyte counts 2 weeks after chemotherapy. These results are similar to our previous experience using *C. parvum* plus chemotherapy in the treatment of metastatic breast cancer (21). Therefore, we cannot agree that *C. parvum* augments hematologic tolerance to chemotherapy.

The discrepancy between our results with *C. parvum* and those of Israel and Edelstein (16) may be due to differences in route, dosage, or schedule of *C. parvum* as well as to differences in chemotherapy, patient population, and possibly other considerations. BCG's lack of efficacy might be attributed to similar phenomena. The sample size, furthermore, is too small to conclude that there can be no modest benefits with those agents. It is possible, however, that putative immunotherapeutic agents such as these are relatively ineffective in the face of a large tumor burden. Regardless, it is clear that iv *C. parvum*, as given in this regimen, was poorly tolerated and not of sufficient value to justify its toxicity.

The combination-chemotherapy regimen MOCA (with or without immunotherapy) was a moderately effective regimen for treating small cell carcinoma. The response rate (68%) and survival (median, 42 weeks) exceed values obtained for single-agent therapy and compare favorably with other combination-chemotherapy and chemotherapy/radiation-therapy regimens (2,3,22). For other histologic types of lung cancer, the response rate to MOCA was not superior to that of other combination-chemotherapy regimens (1,4,5). Survival (either for the entire group or as a function of performance status or response) is comparable to survival seen with other combination regimens (4,5) and is superior to that reported for untreated controls (23). Toxicity of MOCA was substantial but tolerable.

Survival in this series correlated well with performance status. This corroborates the data from Veterans' Administration studies (23) and reinforces the importance of analyzing this variable when assessing the ability of a treatment regimen to prolong survival.

MOCA appears to be of modest value in the treatment of bronchogenic carcinoma, particularly of the small cell type. Immunotherapy with BCG or *C. parvum*, as given in this regimen, did not significantly enhance the effects of MOCA.

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Phase II Trial of Ftorafur With Mitomycin C Versus Ftorafur With Methyl-CCNU in Untreated Colorectal Cancer[1,2]

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SUMMARY

In an attempt to substitute ftorafur for burdensome 5-fluorouracil (5-FU) infusions, 52 previously untreated patients were randomized to receive ftorafur with either mitomycin C or methyl-CCNU. Ftorafur was administered monthly as a 2-hour infusion daily × 5 days. Mitomycin C and methyl-CCNU were repeated every 8 weeks. A response rate of 27% (seven responses among 26 patients) was demonstrated on the mitomycin C arm compared to a response rate of 15% (four responses among 26 patients) on the methyl-CCNU arm ($P = 0.25$). There was no significant difference in the median survival between treatment arms. Central nervous system toxicity occurred in $\geq$ 30% of the patients and appeared to be the limiting factor with ftorafur administration. Alternate schedules of ftorafur should be explored since there appears to be little advantage of a daily ftorafur schedule over conventional 5-FU infusions.

[Cancer Treat Rep 62:689–692, 1978]

Since its synthesis in 1966, the pyrimidine nucleoside ftorafur has been studied extensively and has been found to be active in the following tumor models: Sarcoma 180, Walker 256 carcinosarcoma, alveolar liver cancer PE-1, and Harding-Passey melanoma (1). This 5-fluorouracil (5-FU) analog has been reported to have significant activity in large bowel cancer by Blokhina et al (2). Dose-limiting toxicity of the drug has been central nervous system (CNS) toxicity (3,4) rather than myelosuppression. A phase I study of ftorafur with mitomycin C or methyl-CCNU demonstrated activity with both combinations in colon cancer with acceptable toxicity (3). This initiated a phase II trial of these agents in untreated disseminated colorectal cancer.

MATERIALS AND METHODS

Fifty-two untreated patients with biopsy-proven disseminated adenocarcinoma of the large bowel were entered in the study. All patients had measurable metastatic disease. Patients were randomized to receive ftorafur with either mitomycin C or methyl-CCNU (ftorafur and methyl-CCNU were supplied by the Developmental Therapeutics Program, DCT, NCI, Bethesda, Md). Eligibility requirements included a minimum life expectancy of 8 weeks, a wbc count $> 4000/\text{mm}^3$, a platelet count $> 100{,}000/\text{mm}^3$, a creatinine level < 1.5 mg/100 ml, and informed written consent. Pretreatment evaluation included liver scan and enzymes, chest roentgenogram, and urinalysis. Tumor measurements, serum creatinine, and chemistries, if indicated, were obtained monthly, while cbc and platelet counts were checked weekly.

Twenty-six patients were randomized to receive ftorafur with mitomycin C and 26 patients were allocated to the ftorafur and methyl-CCNU arm. Patients in both arms were demographically comparable as illustrated in table 1. Ftorafur was administered monthly at a dose of 2.5 g/m^2 × 5 days as a 2-hour infusion. Mitomycin C was administered

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TABLE 1.—Demographic comparison of patients

Characteristic	Combination: Ftorafur-methyl-CCNU (26 patients)	Combination: Ftorafur-mitomycin C (26 patients)
Men	14	14
Women	12	12
Liver metastasis	22	21
Palliative surgery	12	13
Curative surgery	14	11
Biopsy only	0	2
Performance status		
Mean	76	75
Median	80	80
Range	50–100	20–100
Metastases at time of diagnosis	10 (38%)	12 (46%)
Age (yrs)		
Mean	63	58
Median	63	60
Range	45–74	42–74

at a dose of 20 mg/m^2 as an iv bolus. Methyl-CCNU was given at a dose of 175 mg/m^2 orally. Mitomycin C and methyl-CCNU were repeated every 8 weeks. Subsequent dosage adjustments of mitomycin C or methyl-CCNU were based on nadir leukocyte and/or platelet counts. The dosage of ftorafur for succeeding courses was reduced if severe CNS toxicity (confusion or marked lethargy) occurred.

A course of therapy was defined as two monthly ftorafur injections with one treatment of either mitomycin C or methyl-CCNU. The number of patients treated per course of therapy is illustrated in table 2.

To be evaluable for a response, a patient must have survived a minimum of 2 weeks after initiating therapy. In all patients, tumor reduction was required for a minimum of 4 weeks to be accepted as a responder.

Twenty-two of the 26 patients receiving the methyl-CCNU combination and 21 of the 26 patients receiving the mitomycin C combination had hepatomegaly with a positive liver scan. A partial remission (PR) in these patients was defined as a decrease of $\geq 30\%$ in the sum of the measurements at both midclavicular lines and the xiphoid.

Four patients receiving the methyl-CCNU combination were evaluated for response by following measurable extrahepatic metastases: abdominal mass, two patients; lymph node, one patient; and lung, one patient. Similarly, five patients receiving the mitomycin C combination were observed in an identical manner: lung, two patients; lymph node, two patients; and perineal mass, one patient. In these patients, a PR was defined as a reduction of $\geq 50\%$ in the sum of the products of the longest perpendicular diameters of all measurable lesions.

The complete disappearance of all known clinical tumor was required for a patient to be classified as a complete responder (CR).

RESULTS

On the ftorafur and mitomycin C arm, 23 of the 26 treated patients were evaluable for response. Two patients died from progressive disease within 14 days of beginning therapy. In one patient, information was insufficient to allow the patient to be evaluable for response. On the ftorafur and methyl-CCNU arm, 21 of 26 treated patients were evaluable for response. Of the five patients not evaluable for response, one died within 2 weeks of starting therapy due to progressive disease, two were considered drug-related deaths, and the remaining two refused further therapy because of CNS toxicity (confusion).

Seven of the 26 (27%) treated patients receiving the mitomycin C combination responded (one CR and six PRs) compared to four PRs among the 26 (15%) patients receiving the methyl-CCNU combination ($P = 0.25$).[4] Of the evaluable patients, seven of 23 (30%) receiving the mitomycin C combination responded versus four of 21 (19%) receiving the methyl-CCNU schedule ($P = 0.30$). Four of the seven responders receiving the mitomycin C arm had

TABLE 2.—No. of patients treated per course of therapy

Drug combination	Course 1	2	3	4	5	6
Ftorafur-methyl-CCNU	26	15	7	3	1	0
Ftorafur-mitomycin C	26	16	11	7	4	1

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[4] All P values were determined by the chi-square or the Student's t test.

TABLE 3.—Myelosuppression (No. of patients)

	Leukopenia grade*						Thrombocytopenia grade†						
	0	1	2	3	4	*P* value	0	1	2	3	4	5	*P* value
Course 1													
Ftorafur-methyl-CCNU	13	5	4	3	0	0.67	13	3	3	3	1	2	0.22
Ftorafur-mitomycin C	9	7	7	0	0		15	3	3	2	0	0	
Course 2													
Ftorafur-methyl-CCNU	10	1	3	1	0	0.84	9	1	3	0	2	0	0.47
Ftorafur-mitomycin C	9	4	3	0	0		11	2	2	1	0	0	
Course 3													
Ftorafur-methyl-CCNU	4	2	0	0	0	0.90	4	1	1	0	0	0	0.95
Ftorafur-mitomycin C	7	4	0	0	0		8	0	3	0	0	0	
Course 4													
Ftorafur-methyl-CCNU	0	1	1	0	0	0.22	1	1	0	0	0	0	0.86
Ftorafur-mitomycin C	4	2	0	1	0		5	0	1	1	0	0	

*0 = no leukopenia; 1 = 3000–4000 cells/mm^3; 2 = 2000–2999; 3 = 1000–1999; 4 = <1000.

†0 = no thrombocytopenia; 1 = 75,000–100,000 cells/mm^3; 2 = 50,000–74,999; 3 = 25,000–49,999; 4 = <25,000; 5 = fatal.

measurable liver metastasis compared to three of the four responders receiving the methyl-CCNU schedule.

No significant difference in the duration of remission was noted between arms for responders (P = 0.41). On the methyl-CCNU arm the duration ranged from 4 to 19 weeks with a mean and median of 9 and 6.5 weeks respectively. Remission duration on the mitomycin C combination ranged from 4 to 30 weeks with a mean and median of 14 and 13 weeks respectively.

SURVIVAL

No significant difference was noted in the median survival between arms for all treated patients. Median survival on the methyl-CCNU arm was 22 weeks compared to 37 weeks on the mitomycin C combination (P = 0.44). The median survival for responders receiving the mitomycin C arm (seven patients) was 44 weeks compared to 24 weeks for nonresponders. This approaches statistical significance (P = 0.08). However, no statistical difference in median survival was noted between responders (four patients) and nonresponders on the methyl-CCNU arm (48 versus 24 weeks) (P = 0.84). There was no significant difference between performance status of responders and nonresponders on either treatment arm.

TOXICITY

For the first course of therapy, the median wbc and platelet counts on the methyl-CCNU arm were 4000 and 110,000/mm^3 respectively compared to the median wbc and platelet counts on the mitomycin C arm of 3600 and 125,000/mm^3 respectively. Through four courses of therapy, ridit analysis demonstrated that neither arm produced significantly greater thrombocytopenia or leukopenia (table 3). In addition, no cumulative myelotoxicity was shown on either arm through four courses.

CNS toxicity occurred in eight of 26 (31%) patients receiving the ftorafur and methyl-CCNU combination compared to ten of 26 (38%) patients receiving the ftorafur and mitomycin C therapy (P = 0.14). Overall, 205 courses of ftorafur were administered, with CNS toxicity occurring in 33 (16%). In all instances, the CNS toxicity began within 2–4 hours of starting the ftorafur infusion. In general, patients remained slightly lethargic through the 5 days of therapy with improvement in mental status observed in all patients the day after ftorafur was discontinued. The CNS toxicity in all but two patients consisted of either lethargy or nervousness. Two patients receiving the methyl-CCNU combination experienced severe confusion and subsequently refused further therapy.

Nausea/emesis was noted in 44% of the patients receiving the methyl-CCNU combination compared to 34% of the patients receiving the mitomycin C combination (P = 0.45).

DISCUSSION

Phase I studies with ftorafur evoked a great deal of interest since the agent produced minimal myelosuppression (5). Pharmacologic studies suggest that ftorafur behaves as a depot form of 5-FU with blood levels similar to those produced by continuous

5-FU infusions (6). 5-FU infusions, which are also nonmyelosuppressive, have been combined with mitomycin C and methyl-CCNU with tolerable toxicity (7,8). Unfortunately, this method of 5-FU administration is burdensome and frequently requires hospitalization. The reported study combined ftorafur with either mitomycin C or methyl-CCNU in an attempt to determine the activity of these combinations in large bowel malignancies and to relieve the patient of the tedious administration of a fluorinated pyrimidine. Although both combinations demonstrated activity (ie, ftorafur and mitomycin C [27%] versus ftorafur and methyl-CCNU [15%]), neither arm was superior in terms of response or survival.

Hematologic toxicity was noted but was tolerable. It is our opinion that the two drug-related deaths on the methyl-CCNU arm do not represent a new toxicity for ftorafur since this phenomenon is similar to that previously described when 5-FU infusions were combined with a nitrosourea (8). Furthermore, the recently concluded phase II trial by the Southwest Oncology Group utilizing ftorafur as a single agent in a daily × 5 schedule did not demonstrate significant bone marrow depression.[5] The other significant side effect noted in this study was the disappointingly high occurrence of CNS toxicity in > 30% of the patients on both treatment arms.

The combination of ftorafur with either mitomycin C or methyl-CCNU does not produce response rates significantly different from those produced by 5-FU used alone or as an infusion in combination with either of the above two agents (8,9).

It appears that little has been gained from replacing 5-FU infusions with ftorafur. However, before final conclusions are made about the efficacy of this drug, it should be mentioned that there are pharmacologic data which show that ftorafur, as a weekly 2-hour infusion, produces sustained blood levels for approximately 48 hours. Perhaps, in an attempt to reduce CNS toxicity, it would seem reasonable to explore this agent in either a weekly or biweekly schedule.

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[5] Minutes of the meeting of the Gastrointestinal Committee, Southwest Oncology Group, New Orleans, La, Feb 2, 1978.

Acute Renal Failure After *cis*-Dichlorodiammineplatinum(II) and Gentamicin-Cephalothin Therapies [1]

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SUMMARY

Combined gentamicin-cephalothin therapy in four patients after treatment with *cis*-dichlorodiammineplatinum(II) (CPDD) for advanced solid tumors was complicated by severe acute renal failure. The total dose of gentamicin varied from 240 to 945 mg and that of cephalothin varied from 28 to 48 g. Low-dose (0.5 mg/kg × 8) CPDD was given to one patient, high-dose (3 mg/kg) CPDD was given to two patients, and very high-dose (5 mg/kg) CPDD was given to one patient. The high and very high doses of CPDD were given with concomitant mannitol diuresis. CPDD therapy was complicated by mild transient azotemia in three patients and by severe acute renal failure in one. In the latter, the azotemia began to improve on Day 7 after CPDD treatment. Following gentamicin-cephalothin therapy, all patients developed severe acute renal failure which persisted until death. At autopsy, all patients had extensive renal tubular necrosis at various stages. These findings indicate that gentamicin-cephalothin therapy after treatment with CPDD can be severely nephrotoxic, and that this antibiotic combination should be given with great caution, if at all, to patients receiving CPDD treatment for malignancy.

[Cancer Treat Rep 62:693–698, 1978]

Impairment of renal function is the main toxic side effect of therapy with *cis*-dichlorodiammineplatinum(II) (CPDD), one of the newer anticancer drugs (1–3). The renal lesions caused by the drug in man are now well-characterized (4,5). The induction of osmotic diuresis with mannitol concomitantly with the administration of CPDD has considerably ameliorated the deleterious effect of this compound on the kidneys (4,6,7).

A potential for renal damage is known to exist with the widely used broad-spectrum antibiotic combination of gentamicin and cephalothin (8–14). We have observed that the administration of this antibiotic combination following CPDD therapy enhanced the renal insufficiency. The purpose of this paper is to report on these observations.

MATERIALS AND METHODS

Four patients with advanced solid tumors treated with CPDD as the last anticancer agent prior to death were selected for this study. None of these patients had underlying renal disease or previous treatment with nephrotoxic drugs. This was attested to by normal BUN and serum creatinine levels in all four patients prior to therapy with CPDD.

CPDD, manufactured by Ben-Venue Laboratories, Inc, Bedford, Ohio, was supplied by the National Cancer Institute, Bethesda, Md, in vials containing 10 mg of CPDD, 100 mg of mannitol, and 90 mg of sodium chloride. It was reconstituted with 10 ml of sterile water for injection and was administered iv over a 10–20-minute period. The dose schedules of CPDD are listed in table 1. The patients treated with high and very high doses of CPDD were also

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TABLE 1.—Clinical data of patients treated with CPDD and the antibiotic combination of gentamicin-cephalothin

Patient No.	Age (yrs)	Race/sex	Tumor type	Dose schedule			Survival in days after last dose of CPDD
				CPDD	Gentamicin	Cephalothin	
1	30	Black/F	Carcinoma, mammary duct	3 mg/kg (160 mg), high-dose	40 mg q8hrs from Day 3 after CPDD; total, 240 mg	2 g q4hrs from Day 1 after CPDD; total, 48 g	5
2	67	White/M	Epidermoid carcinoma, pharynx	3 mg/kg × 2 (310 mg), high-dose	100 mg on Day 13 after CPDD, 50 mg q12hrs from Day 13 after CPDD; total, 945 mg	2 g q6hrs from Day 13 after CPDD; total, 28 g	15
3	33	White/F	Hodgkin's disease	0.5 mg/kg × 8 (155 mg), low-dose	45 mg q8hrs from Day 0 after last dose of CPDD; total, 945 mg	2 g q6hrs from Day 0 after last dose of CPDD; total, 32 g	15
4	55	White/M	Adenocarcinoma, rectum	5 mg/kg (400 mg), very high-dose	120, 70, and 85 mg on Days 14, 17, and 21 after CPDD respectively; total, 275 mg	2 g q6hrs from Day 12 after CPDD; total, 46 g	23

given mannitol to induce diuresis according to a protocol described elsewhere (7).

Gentamicin (Schering Corporation, Kenilworth, NJ) and cephalothin (Eli Lilly and Co, Indianapolis, Ind) were given to all four patients because of bacterial infection at various intervals of time after CPDD therapy (table 1). The renal function was assessed by daily BUN and serum creatinine levels. Serum levels of the antibiotics or of CPDD were not measured.

A complete autopsy was performed on all patients with the exclusion of the brain in two patients. Multiple kidney sections, stained with hematoxylin and eosin, were studied in every case.

RESULTS

Clinical Findings

Table 1 lists in detail the patients' age, sex, race, tumor types, dose schedules, and survival time after CPDD therapy. Figures 1–4 illustrate the clinical course of every patient in relation to CPDD and gentamicin-cephalothin therapies.

The patients treated with low-dose CPDD and high-dose CPDD plus mannitol diuresis developed mild transient elevation of BUN and/or serum creatinine levels following administration of the platinum compound and prior to antibiotic therapy (figs 1–3).

Severe acute renal failure occurred in patient 4 soon after very high doses of CPDD plus mannitol therapy (fig 4). This patient also exhibited other complications of CPDD therapy, ie, pancytopenia and deafness (1–3). The BUN and serum creatinine levels in this patient began to decrease on Day 7 after CPDD therapy and were returning to normal when the antibiotics were started (fig 4).

In all patients, treatment with gentamicin and cephalothin (following CPDD therapy) was complicated by severe progressive acute renal failure which persisted until death (figs 1–4).

Pathologic Findings

The kidneys were enlarged in all four patients, having an average weight of 220 g. The renal cortices were pale and swollen and the medullae exhibited marked congestion.

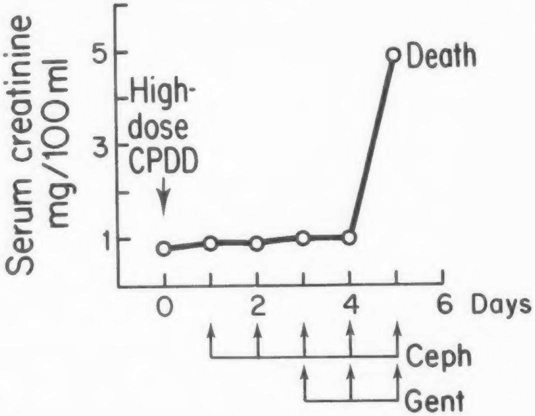

FIGURE 1.—Serum creatinine levels of patient 1 in relation to CPDD and gentamicin-cephalothin.

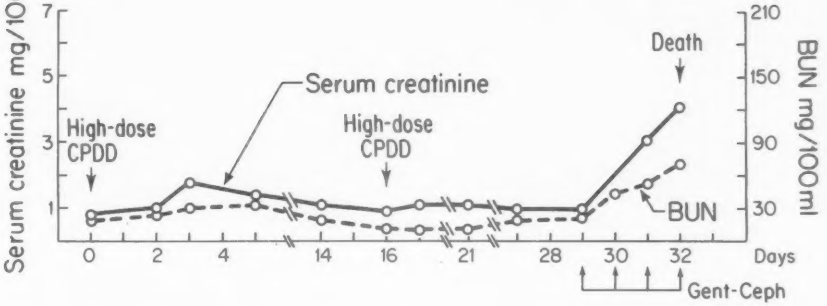

FIGURE 2.—Renal function of patient 2 following treatment with CPDD and gentamicin-cephalothin.

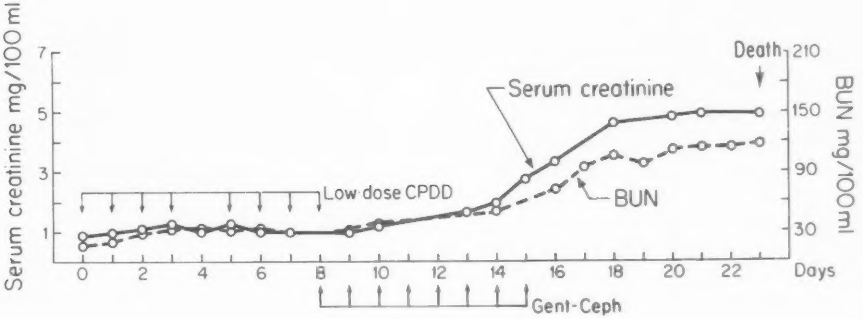

FIGURE 3.—BUN and serum creatinine levels of patient 3 in relation to treatment with CPDD and gentamicin-cephalothin.

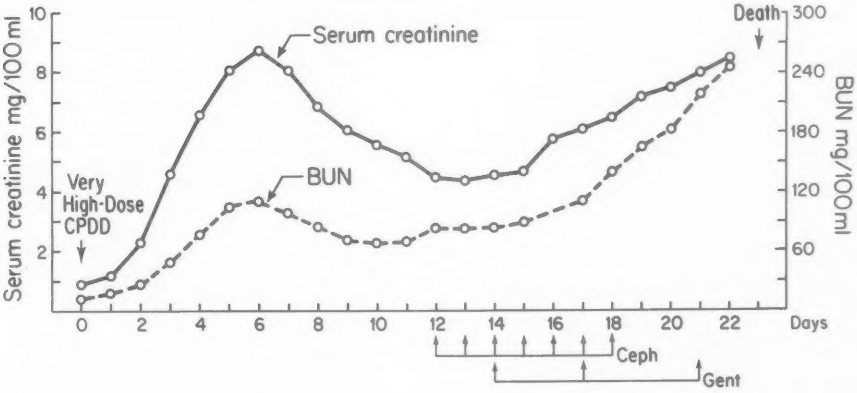

FIGURE 4.—Renal function of patient 4 in relation to treatment with CPDD and gentamicin-cephalothin.

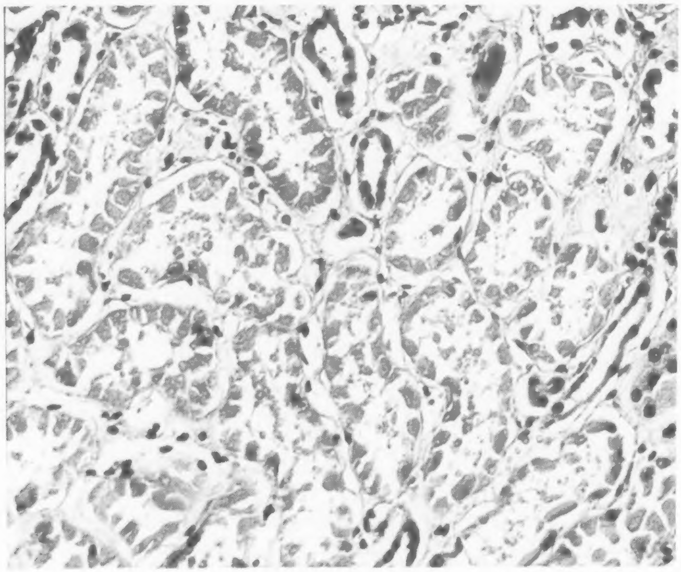

FIGURE 5.—Renal changes associated with gentamicin-cephalothin therapy following treatment with high-dose CPDD (patient 1). There is extensive acute tubular necrosis involving both proximal and distal convoluted tubules (H & E stain, × 300).

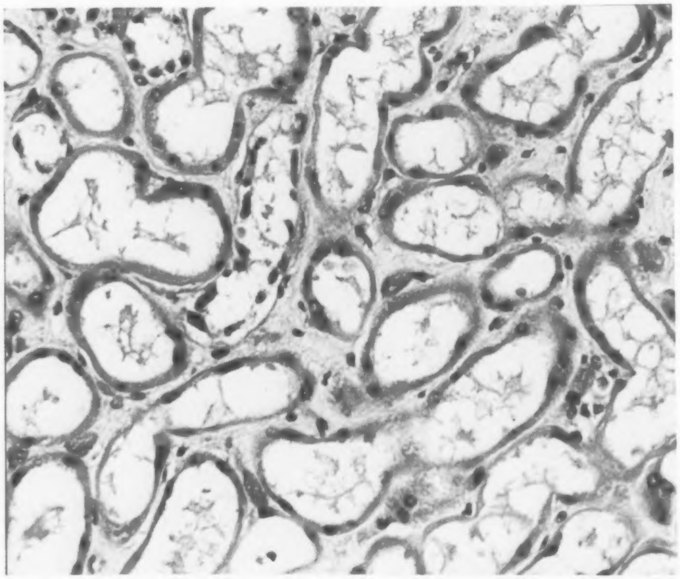

FIGURE 6.—Renal changes associated with gentamicin-cephalothin therapy following administration of high-dose CPDD (patient 4). The convoluted tubules are markedly dilated with flattening of epithelium and increased cytoplasmic basophilia. There is proteinaceous material in the tubular lumina and the interstitium is markedly edematous (H & E stain, × 300).

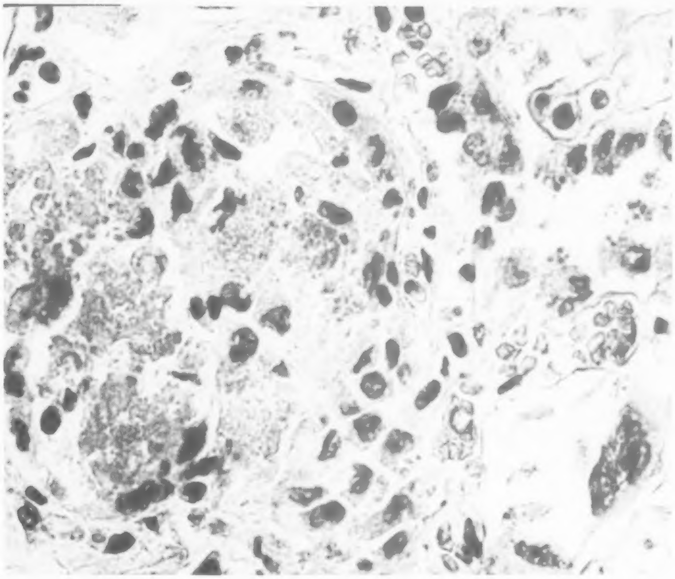

FIGURE 7.—Renal changes associated with gentamicin-cephalothin therapy after treatment with low-dose CPDD (patient 3). A convoluted tubule contains necrotic debris in its lumen, and elsewhere, atypical tubular epithelial cells are seen (H & E stain, × 610).

Microscopically, in all patients the kidneys showed extensive acute tubular necrosis involving both proximal and distal tubules (fig 5). Necrosis of collecting ducts was also present in patient 3. Dilated proximal and distal tubules, lined by flattened epithelium showing increased cytoplasmic basophilia, were also observed in patients 2 and 4 (fig 6). Cellular atypia of convoluted and collecting tubular epithelium was apparent in three patients but was most striking in patient 3 (fig 7). Other changes common to all patients included numerous hyaline and granular casts, interstitial edema, and congestion of vasa recta. The glomeruli did not show any significant change except for occasional atypical nuclei in patient 4.

DISCUSSION

The biochemical and autopsy findings in all four patients indicate that a significant damage of the renal tubules had ensued in the wake of therapy with CPDD and the antibiotic combination of gentamicin and cephalothin. CPDD is a known nephrotoxic compound in both experimental and clinical settings (1–4). The nephrotoxicity of CPDD is roughly dose-related (1,2) and may be significantly ameliorated by concomitant induction of diuresis with mannitol (4,7). Our clinical findings appear to confirm these observations.

Renal pathologic changes induced by CPDD therapy may persist up to 4 weeks after administration of this compound even though the BUN and serum creatinine levels have returned to normal (4).

Gentamicin nephrotoxicity has been well-demonstrated in experimental animals (15,16). Administration of this agent to rats elicits acute tubular necrosis, primarily affecting the proximal convoluted tubules (15,16). Although it has been asserted that gentamicin has little, if any, nephrotoxicity in man (12,17,18), there are many reports to the contrary (15,19–21).

Cephalothin has been reported to be nephrotoxic when given in high doses to patients with (22–25) or without (26) pre-existing impairment of renal function.

Simultaneous administration of cephalothin and gentamicin to rats has been shown to prevent the nephrotoxicity of the latter (27). However, numerous clinical studies have reported acute tubular necrosis or other evidence of renal failure associated with combined gentamicin-cephalothin therapy (8–14,28–31). These findings suggest that the combination of gentamicin and cephalothin in man might be more nephrotoxic than either of these antibiotics alone.

Our results suggest that low-dose CPDD and high-dose CPDD with mannitol diuresis may cause a mild transient impairment of renal function while very high doses of CPDD (5 mg/kg) can induce severe renal failure despite mannitol diuresis.

Our findings also indicate that when gentamicin and cephalothin are administered to patients with underlying renal tubular damage induced by CPDD, acute renal failure may ensue.

The pathologic changes found in our patients were essentially similar to those described in patients treated with gentamicin-cephalothin therapy (10,11), and significantly, they were also similar but much more extensive than those seen in patients treated with CPDD alone (4).

It would appear, therefore, that cephalothin and gentamicin renal toxicity augments CPDD tubular injury and for this reason these antibiotics should be given with great caution, if at all, to patients receiving CPDD for malignancy.

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Intra-Arterial Radionuclide Infusion: A New Technique to Assess Chemotherapy Perfusion Patterns [1,2]

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SUMMARY

A mechanical infusion pump was used to introduce ^{99m}Tc-sulfur colloid at a rate of 1 ml/minute into the hepatic arteries of six patients. The radiotracer distribution patterns were compared to those obtained with "bolus" radionuclide angiography and those seen with standard contrast angiography. In all cases, the slow flow rate radionuclide studies evidenced dramatic differences from the patterns obtained with rapid flow rate injections. The results indicate that standard, rapid-injection contrast techniques may not produce the most reliable information regarding potential flow distribution. It is concluded that radiotracers introduced at flow rates approximating those attained with infusion pumps will offer the best estimates of both initial catheter placement and subsequent patterns of hepatic distribution of chemotherapeutic agents.

[Cancer Treat Rep 62:699–703, 1978]

Cancer metastatic to the liver carries a poor prognosis; in the absence of therapy, the overall median survival is 2.5 months (1). Hepatic metastases treated with oral and iv chemotherapeutic agents demonstrate a 20% response rate but no significant increase in survival (2). Attempts to improve these statistics have included intrahepatic arterial drug infusions (3). The rationale for this mode of therapy rests with the presumed ability of the liver to detoxify high concentrations of these agents. This results in diminished bone marrow and gastrointestinal toxic effects while permitting high levels of the drug to reach tumor cells. Response rates of 50%–60% with associated prolongation of survival have been reported (4–6).

In the past, high flow rate radionuclide and contrast angiography (7,8) have been used to assess catheter placement and vascular anatomy in patients receiving intra-arterial infusions. This study was undertaken to evaluate the effect of varying infusion rates on the distribution of radionuclide and contrast agents. It was hoped that this investigation would be of assistance in determining the intrahepatic distribution of chemotherapeutic agents introduced at the flow rates routinely used for hepatic artery infusions.

MATERIALS AND METHODS

Six patients with surgically proven primary or secondary hepatic malignancies were studied (table 1). In all patients a 7-French catheter (2.3 mm outer diameter) was positioned in the common hepatic artery. The left axillary arterial approach was used in four patients, the right femoral artery in one patient, and an intraoperative placement of a common hepatic artery catheter in the sixth patient. After initial catheter localization, hepatic artery anatomy was studied by injecting contrast at flow

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TABLE 1.—Summary of patient data

Patient No.	Sex	Age	Tumor	Chemotherapy
1	M	65	Colon	5-FUDR
2	M	56	Hepatoma	5-FUDR
3	F	53	Colon	5-FUDR
4	F	43	Colon	5-FUDR
5	F	38	Breast	5-FUDR
6	F	58	Colon	5-FUDR

rates of 6–8 ml/second with filming at a rate of two frames per second.

Within 48 hours of catheter placement the patients were again studied by both angiography and radionuclide perfusion; in all cases both examinations in the re-evaluation were performed within a 2-hour interval.

Approximately 4 millicuries of ^{99m}Tc-sulfur colloid or ^{99m}Tc-stannous phytate (9) (both obtained from New England Nuclear Corp, Boston, Mass), in a volume of 0.2 ml, was injected into the catheter through a three-way stopcock. A portable infusion pump (Model 922, Imed Corp, San Diego, Calif) was attached to the end of the catheter and was utilized to introduce the radiotracer at a flow rate of 0.017 ml/second (1.0 ml/minute).

Imaging was performed using a large-field-of-view gamma camera with a 140-KeV low energy all-purpose parallel hole collimator centered over the abdomen. Recording was done on radiographic film using a multi-imaging device preset to collect 80 frames of information at a time interval of two frames per second. In order to assess radiocolloid distribution as a function of initial hepatic artery blood flow, only the initial 10 seconds of images were evaluated. Hepatic and splenic uptake seen subsequent to this reflected reticuloendothelial sequestration of recirculating colloid and was not considered.

Following this "slow" infusion, the pump was disconnected and an additional 8 millicuries of radionuclide was placed in the catheter via the three-way stopcock. A rapid hand flush with 15 ml of saline provided a bolus injection, rather than a constant infusion, of the tracer to simulate flow rates achieved during angiography. Imaging and recording were identical to those used during the slow infusion procedure.

Following radionuclide infusion, contrast angiography was performed using flow rates of 4 and 1 ml/second, significantly slower than the 6–8 ml/second initially utilized.

RESULTS

The results are summarized in table 2. A striking difference in distribution was exhibited when rapid flow rates were compared with slow flow rates. This difference was seen with both contrast and radionuclide angiography (figs 1 and 2).

Where the contrast distributions were compared with those obtained with radionuclides the following were observed: at fast flow rates the two tracers behaved similarly in all patients and at slow flow rates they agreed in four instances and disagreed in two (patients 3 and 4).

No differences were found between the distribution of contrast agents injected at 4 or 8 ml/second.

TABLE 2.—Comparison of contrast and radionuclide angiography at fast and slow infusion rates

	Contrast angiography*		Radionuclide angiography*	
	Infusion†		Infusion†	
Patient No.	Fast (4.0)‡	Slow (1.0)	Fast (~ 1.0)	Slow (0.017)
1	R L S	R L	R L S	R L
2	R L S	R	R L S	R
3	R L	R	R L	L>R
4	L:S>R	L S	L:S>R	L>S>R
5	R L G-D	R L	R L G-D	R L
6	L>S>R	R>L	L>S>R	R>L

*R = right lobe of liver perfused; L = left lobe of liver perfused; S = spleen perfused; and G-D = gastroduodenal artery perfused.

†: = identical perfusion and > = greater organ perfusion.

‡Nos. in parentheses = infusion rate in ml/sec.

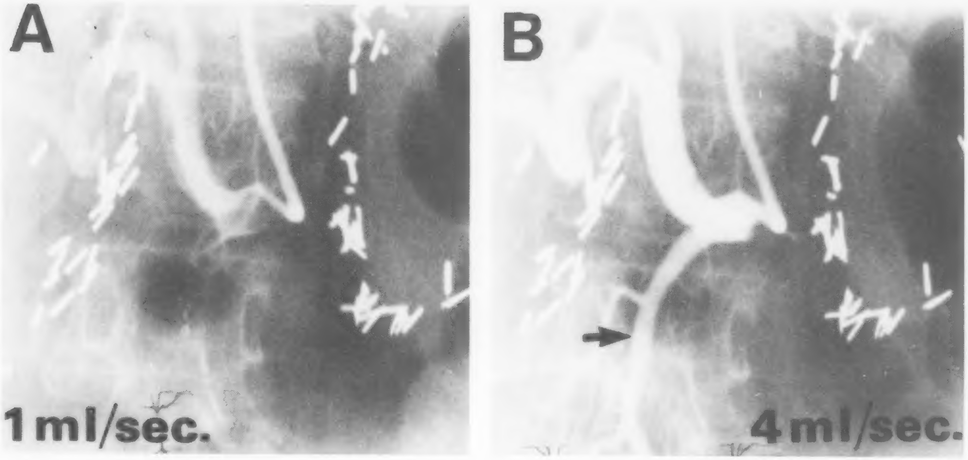

FIGURE 1.—A. Contrast agent, administered at a rate of 1.0 ml/sec, opacifies the common hepatic artery and its major branches. B. At higher flow rates (4.0 ml/sec) filling of the gastroduodenal artery (arrow) is seen.

DISCUSSION

We feel that it is important to determine the distribution of chemotherapeutic agents when they are infused into the liver. If much or most of the agent is delivered to non-tumor-bearing areas, ie, uninvolved portions of the liver, gastrointestinal tract, and spleen, then the therapeutic effect would be no different from that of an iv injection and the advantages of intrahepatic arterial infusion would be negated.

The higher flow rates routinely employed during contrast angiography produce distribution patterns which differ significantly from those patterns seen at lower infusion rates. Indeed, the slowest contrast flow rates which allowed interpretable vascular

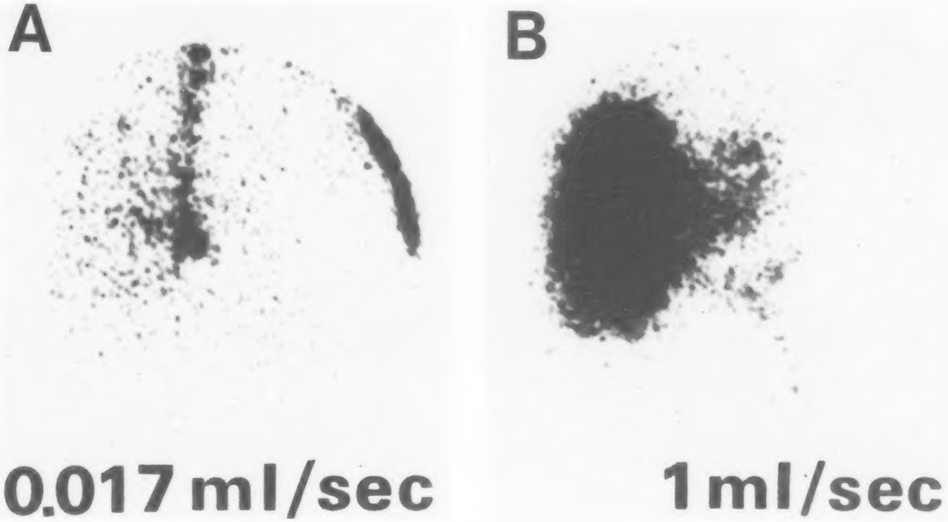

FIGURE 2.—A. Radiocolloid, administered at a rate of 0.017 ml/sec, is seen in the 0.5-sec image to be deposited mainly within the right lobe of the liver. B. Rapid infusion (approximately 1 ml/sec) of the radiocolloid seen in the 0.5-sec image shows tracer entering the right and left lobes of the liver as well as the gastroduodenal artery.

definition (1 ml/second) in two of six patients produced distribution patterns differing from the even slower radionuclide infusions (0.017 ml/second).

Disparate distribution patterns seen during investigations of hepatic blood flow have relied primarily upon the principles of turbulent versus streamlined flow to explain the observed results (10–12). Although the disruption of streamlined flow may play a role, it is more likely that the variations in the patterns of distribution can largely be accounted for by the relative increases in flow rate within the hepatic artery as a result of the infusions, and the associated pressure increases.

It has been shown that during selective arterial injection of contrast or other fluid, the flow rate within that artery is substantially increased (13). The degree of this increase varies as a function of the injection rate through the catheter, the relative lumen size of the catheter, and the vessel into which it has been positioned.[5]

As a hypothetic example, if one assumes a nominal hepatic artery flow rate of 3–10 ml/second (14,15) and a constant injection rate of 8 ml/second, the total flow rate during the injection could reasonably be expected to rise to approximately 18 ml/second. The intra-arterial increase in flow is associated with a significant pressure increase within the artery during the period of injection. Part of this pressure increase is accommodated by the distensibility of the arterial wall, allowing a portion of the injected bolus to become temporarily stored within that segment of the artery; part of the pressure increase is used to overcome the augmented resistance to the increased flow offered by the distal capillary bed. If the pressure rate increase is sufficient, some retrograde flow will occur, thus accounting for the observed back flow and side flow into the splenic, gastroduodenal, and initially nonvisualized hepatic artery. Low infusion rates, compared to the rates of normal blood flow, produce pressure increases so small that retrograde flow does not occur.

The disparity between radionuclide and contrast angiography at varying flow rates (table 2) can also be accounted for on the basis of the agents employed. Unlike an intra-arterial injection of contrast material which is subject to rapid dilution and dissipation, the initial distribution of radiocolloid, administered by hepatic artery catheter, is a reflection of first pass deposition and rapid phagocytosis by the reticuloendothelial system. This difference in deposition can account for the increase in anatomic detail seen with fast infusion radionuclide angiography when compared to slow infusion contrast angiography, in spite of the fact that both studies are performed at 1.0 ml/second. The extremely slow flow achieved with the infusion pump allows a greater opportunity for first pass hepatic phagocytosis of radiocolloid and produces similar increased anatomic detail even with flow rates of 0.017 ml/second.

Our observations indicate that during hepatic artery catheter placement for infusion of chemotherapeutic agents, radiocolloid and contrast media should be injected at flow rates approximating those of infusion pumps. Specifically, the use of small quantities of ^{99m}Tc-labeled radiocolloid introduced at flow rates of 1 ml/minute to approximate therapeutic infusion, appears to allow the best estimate of both catheter placement and subsequent patterns of intrahepatic drug infusion.

An additional application is related to thrombus formation at the catheter tip which can result in compromised drug flow. This technique of radionuclide infusion allows a rapid and simple method for the reassessment of potentially altered distribution patterns.

Other efforts currently under investigation include the use of ^{99m}Tc-macroaggregated albumin rather than sulfur colloid as the radio-indicator. This has allowed a better appreciation of first pass perfusion patterns since with capillary blockade, greater quantities of radiotracer are trapped within the liver initially, and the subsequent hepatic and splenic reticuloendothelial uptake of recirculating colloid is obviated. Additionally, the intrahepatic distribution of radiolabeled macroaggregated albumin should provide a map of relative hepatic artery regional flow. Also, we will attempt to correlate slow flow distribution patterns with tumor response as this will offer the ultimate validation of the utility of this approach.

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Chemotherapy of Transplantable Mouse Tumors With *cis*-Dichlorodiammineplatinum(II) Alone and in Combination With Sarcolysin [1]

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SUMMARY

The therapeutic properties of *cis*-dichlorodiammineplatinum(II) (DDP) were studied in 12 strains of transplantable tumors and leukemias of mice. It was shown that this compound possesses a wide spectrum of antitumor action. Under its influence, the growth of solid tumors of certain strains is markedly inhibited. The best effects were obtained in treating adenocarcinoma of the large intestine (strain AKATOL), cancer of the forestomach (strain PRG), Sarcoma 180, and mammary Adenocarcinoma 755 (Ca 755). The lifespan of mice with leukemia (strains L1210 and La), plasmacytoma MOPC-406, and ascitic Hepatoma 22 was considerably increased. Animals with L1210 leukemia were in individual instances completely cured. In three tumor strains (AKATOL, Ca 755, and Sarcoma 37) a much greater antitumor effect was obtained when DDP was given in combination with sarcolysin than when the drugs were used individually. Enhanced antineoplastic action was especially noticeable in the increase in the number of completely regressed tumors of strain AKATOL and Sarcoma 37. Histologic studies of Ca 755 during chemotherapy showed that immunocompetent cells of the host play a prominent role in the mode of antineoplastic action of the platinum compound. This is manifested by the development of an intense lymphohistiocytic reaction around and inside the treated tumors. The main side effects of the drug are lesions of the convoluted tubules of the kidneys and villi of the small intestine.

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Analyzing numerous papers from the USSR and elsewhere, published prior to 1947 and devoted to the study of antitumor properties of various metal compounds, Larionov and Mankin (1) and Vermel (2) noted that results with these agents were unsatisfactory. Nevertheless, they concluded that a search for antineoplastic agents among inorganic and metallo-organic compounds should be continued.

After World War II, the efforts of most chemists and chemotherapists were directed to the development of other avenues of cancer chemotherapy, namely, alkylating agents, antimetabolites, antibiotics, and plant alkaloids (3). Because new trends in tumor chemotherapy looked more promising, the study of metallic compounds for the treatment of malignant neoplasms was relegated to second place.

In 1969, Rosenberg et al (4) demonstrated considerable antineoplastic activity of *cis*-dichlorodiammineplatinum(II) (DDP). This paper renewed the interest of chemotherapists in inorganic and bioinorganic agents, especially various platinum compounds. After thorough study, DDP was submitted to clinical trials. Although DDP has already demonstrated definite practical usefulness, it is still being intensely scrutinized under experimental conditions from many different aspects (5–12). This is explained by the peculiarities of the antitumor effect of DDP which is not typical for antineoplastic drugs of other classes, for instance, the alkylating agents.

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TABLE 1.—Tumor strains used in the study

Tumor strain	Strain of mice
AKATOL, adenocarcinoma of the large intestine	BALB/c
PRG, squamous cell carcinoma of the forestomach	CC57Br
OG-5, squamous cell carcinoma of the forestomach	C3HA
RSchM-5, squamous cell carcinoma of the uterine cervix	CBA
Ca 755, mammary adenocarcinoma	BDF_1 (C57BL × DBA/2), hybrid mice of the 1st generation
Lewis lung carcinoma	BDF_1
Hepatoma 22, ascitic form	BDF_1
L1210 lymphoid leukemia	BDF_1
La hemocytoblastic leukemia	BDF_1
Plasmacytoma MOPC-406	BALB/c
Sarcoma 180	Noninbred white mice (SHY/K_v)
Sarcoma 37	Noninbred white mice (SHY/K_v)

This paper reports on the results of experimental studies of DDP action against a wide spectrum of transplantable tumors, including strains obtained in the USSR. Data on peculiarities of the cytotoxic effect of the compound on tumor and normal tissues are given. Based on differences in the mode of antitumor action and effect on normal tissues, we also studied combination chemotherapy of tumors with DDP and sarcolysin.

MATERIALS AND METHODS

All experiments were performed on mice weighing approximately 20 g. The strains of transplantable tumors used are shown in table 1. Solid tumors were inoculated sc in the region of the anterior axilla. Leukemias, plasmacytoma MOPC-406, and ascitic Hepatoma 22 were transplanted ip. Each mouse received 10^6 leukemia or ascitic Hepatoma 22 cells. The following method of transplantation of solid tumors was used. After aseptic removal from the animal, the tumor was cut and washed in Medium 199. Tissue free from necrosis was carefully shredded by scissors. Suspension was prepared with 1 g of tumor tissue to 10 ml of Medium 199. The suspension (0.5 ml) was injected sc into each mouse by syringe with a needle 1.0 mm in diameter. The same method of transplantation was used for MOPC-406. In this case, the intraperitoneal lymph nodes affected by the plasmacytoma were shredded and a tissue suspension was prepared. The MOPC-406 was implanted ip. All tumor strains were obtained from the tumor strain bank of the Cancer Research Center, AMS USSR.

Each experimental and control group consisted of six to eight animals. A 50% tumor growth inhibition and a 25% increase in lifespan of mice with leukemias and ascitic tumors were considered as minimum criteria of drug activity.

DDP was dissolved in saline and administered ip. Treatment was begun 24 hours after transplantation of leukemias, 48 hours after implantation of solid tumors and plasmacytoma MOPC-406, and 72 hours after transplantation of ascitic Hepatoma 22. Three treatment regimens were used: (*a*) single maximum tolerated dose (MTD), (*b*) daily injections for 5 days, and (*c*) large doses with 3–5-day intervals (*see* tables 2 and 3).

Therapeutic effect was assessed by the increase in lifespan of mice with leukemias, plasmacytoma, and ascitic Hepatoma 22. The percentage of growth inhibition of solid tumors was calculated by measuring the tumor volume at different periods of treatment and after the end of therapy according to the following formula:

$$\frac{V_c - V_e}{V_c} \times 100\%$$

where V_c = average tumor volume in the control group and V_e = average tumor volume in the experimental group. Tumor volume (mm^3) was determined by multiplying the three diameters. After mice were killed, the following formula was used:

$$\frac{W_c - W_e}{W_c} \times 100\%$$

where W_c = average tumor weight in the control group and W_e = average tumor weight in the experimental group. The methods of screening anticancer drugs in the USSR are described in detail by Larionov (13) and Sofina (14).

Combination chemotherapy of tumors with DDP and sarcolysin was tested on three strains of neoplasms: AKATOL, Adenocarcinoma 755 (Ca 755), and Sarcoma 37. In these experiments, drugs were used in doses equal to one half of the MTD in a single ip injection. The treatment schedule was as follows: sarcolysin was dissolved in saline and administered ip 6 hours after ip injection of DDP; injections were repeated 1 week later. Preliminary experiments proved that shortening the interval between injections (ie, reducing the in-

TABLE 2.—Effect of DDP on the growth of various solid tumors

Strain of tumor	Dose (mg/kg)	Treatment days	Tumor weight inhibition* (%)
AKATOL	1.5	2–6	72
	2.5	2–6	85
PRG	2.5	2–6	85
RSchM-5	0.8	2–6	53
OG-5	2.5	2–6	34
Ca 755	8.0	2	95
	3.0	2–6	60
Lewis lung carcinoma	8.0	2	53
	3.0	2–6	59
Sarcoma 180	3.0	2–6	70
	5.0	2, 5, 8, 11	80
Sarcoma 37	2.5	2–6	59

*Tumor weight inhibition was calculated on the 10th day after treatment.

terval from 7 to 4 days) markedly enhanced toxicity of the drugs and led to death in most of the animals. Combination chemotherapy was begun 48 hours after tumor inoculation. In all therapeutic tests, six to eight animals were used for each dose of the drugs (*see* table 4). Mice showing complete regression of tumors were kept under observation for > 2 months to document relapses.

Pathomorphologic studies of changes in normal tissues were undertaken in noninbred white mice (SHY/K_v), free from tumors, on Days 1, 2, 4, 5, 7, 9, 12, and 15 after a single ip injection of the MTD of DDP (8 mg/kg) and the MTD of sarcolysin (12 mg/kg). Both drugs were dissolved in saline and administered ip. The following organs were examined macroscopically and microscopically: duodenum, small intestine, colon, kidneys, liver, lungs, heart, adrenals, testis, thymus, axillary lymph nodes, and femoral bone marrow. Organs were fixed in 10% neutral formaldehyde and imbedded in paraffin, and sections were stained with hematoxylin and eosin.

TABLE 3.—Antitumor activity of DDP against leukemias and ascitic Hepatoma 22

Strain of tumor	Dose (mg/kg)	Treatment days	ILS (%)
L1210	1.0	1–5	50
	2.0	1–5	60
	3.0	1–5	116
	4.0	1–5	142*
La	8.0	1	270
	2.5	1–5	200
MOPC-406	6.0	2	100
	8.0	2	112
	1.5	2–6	63
	2.0	2–6	144
Ascitic Hepatoma 22	5.0	3	120
	1.5	3–8	26

*2 of 6 mice were alive 60 days after the end of treatment; when killed, no ascitic fluid was noted.

The study of the cytotoxic action of DDP on tumor tissue was carried out during treatment of mice with Ca 755 by either a single injection of the MTD or by a 5-day course of injections at a dose of 3 mg/kg. To detect differences in the cytotoxic effect of DDP and sarcolysin on tumor tissue, an analogous histologic study was undertaken with sarcolysin treatment using a single ip injection of the MTD or a dose of 4 mg/kg/day for 5 days. Sections of tumors were also stained with hematoxylin and eosin. The changes in tumor and normal tissues of mice with Ca 755 at different periods after a single injection of DDP alone and sarcolysin alone at the MTD were also studied.

RESULTS

DDP has considerable toxicity. After ip injection of the drug to noninbred male mice (SHY/K_v), the LD50 is 12 mg/kg and the single MTD is 8 mg/kg. The daily therapeutic dose of DDP with a 5-day course of treatment is approximately 2.5 mg/kg, but can vary widely depending on the animal and tumor strain. Thus, BDF$_1$ mice with L1210 lymphoid leukemia will tolerate daily injections for 5 days of 4.0 mg/kg of the drug, but the same mice with implanted Ca 755 show an MTD of 3.0 mg/kg with the same course of treatment. Very poor tolerance of the drug is displayed by CBA mice inoculated with cancer of the uterine cervix (RSchM-5) where the MTD with the 5-day therapy regimen is only 0.8 mg/kg.

Data on the antitumor activity of DDP given at the MTD are shown in tables 2 and 3. DDP has a wide spectrum of antitumor activity; it considerably inhibits the growth of solid tumors of certain strains. The most sensitive tumor strains were adenocarcinoma of the large intestine (strain AKATOL), cancer of the forestomach (strain PRG), Sarcoma 180, and breast adenocarcinoma (strain Ca 755). Growth of solid tumors of other strains was inhibited to a lesser extent. The lifespan of mice with hemocytoblastosis La, L1210 lymphoid leukemia, and plasmacytoma MOPC-406 is markedly enhanced after DDP treatment. In some experiments, complete cure of animals with L1210 has been recorded. The drug also noticeably pro-

TABLE 4.—Antitumor activity of DDP and sarcolysin administered individually and in combination

		AKATOL		Ca 755		Sarcoma 37	
Drugs	Schedule*	Tumor growth inhibition† (%)	No. of mice with complete tumor regression/No. of treated mice	Tumor growth inhibition (%)	No. of mice with complete tumor regression/No. of treated mice	Tumor growth inhibition (%)	No. of mice with complete tumor regression/No. of treated mice
DDP	4.0/7 × 2	50	0/8	40	0/6	46	2/6
Sarcolysin	6.0/7 × 2	60	0/8	55	0/6	46	2/6
DDP + sarcolysin	4.0/7 × 2 6.0/7 × 2	96	7/8	90	0/6	89	5/6

*Dose (mg/kg)/interval (days) × No. of injections.

†Tumor growth inhibition was calculated after the tumor volume on the 10th day after the 2nd injection of drugs.

TABLE 5.—Duration of antitumor activity of DDP

		Tumor growth inhibition (%)		
		No. of days after the end of treatment		
Strain of tumor	Schedule*	2	7	14
Ca 755	1.5/24 × 5	92	85	52
	3.0/24 × 5	95	78	60
Sarcoma 180	3.0/24 × 5	76	82	70
	5.0/72 × 4	73	93	80

*Dose (mg/kg)/interval (hrs) × No. of injections.

longs the lifespan of mice with ascitic Hepatoma 22. The antitumor effect of DDP is retained for a long time after the end of treatment (table 5). Much greater therapeutic effect was obtained when DDP was given in combination with sarcolysin than when the drugs were used individually (table 4). Enhancement of the antitumor action of DDP was especially evident by the increased number of cured tumors of the AKATOL strain and Sarcoma 37. Observation for a long period (> 2 months) of animals with tumor regression showed no sign of tumor relapse, ie, complete cure has apparently taken place. In these experiments there was no sign of potentiation of toxicity of the two drugs used according to the scheme of combination treatment described above.

The success of combination cancer chemotherapy, in our opinion, is explained by the fact that there exist essential differences in the mechanisms and manifestations of cytotoxic action of the alkylating agent and of the platinum coordination complex on tumor and on normal tissues.

In contrast to sarcolysin, DDP produces in Ca 755 a powerful lymphohistiocytic reaction which embraces the loose connective tissue around the treated tumor and its stroma. This reaction occurs rapidly after the DDP injection, namely, in 24 hours after a single injection of the MTD and after two to three injections of the drug at the daily therapeutic dose. The earliest effect is dilatation of the blood vessels around the tumor and emergence of lymphocytes and leukocytes from the vascular bed into the extravascular spaces. There is activation of lymphohistiocytic elements in the connective tissue around the tumor and its stroma with an increase in the number of mitoses. These activated proliferating lymphohistiocytic elements penetrate the tumor layers (forming at times powerful bands and accretions), push apart and surround the tumor cells, and come into close contact with the tumor cells (figs 1 and 2). Tumor cells undergo deep dystrophic changes, especially in the cytoplasm (vacuolization, destruction of structures), which produce cell death. As a result of the destruction of tumor cells, the neoplastic tissue thins down and forms cavities of varying dimensions, filling with macrophages. Cellular detritus is actively phagocytized by macrophages in all the areas of the treated tumor. Stromal fibrosis at the sites of the destroyed tumor cells goes through the stage of granulation tissue.

Besides its peculiar action on the tumor tissue, DDP shows certain side effects which are not seen with sarcolysin and other alkylating agents. Most of all, this concerns its action on the small intestine, kidneys, and liver.

Twenty-four hours after administration of the single MTD of DDP to mice, epithelial cells of the villi apices degenerate and peel off (fig 3). The main effect of sarcolysin is the destruction of mitotically dividing crypt cells which results in sedimentation of the villi into the submucous layer. Four to 5 days after the single MTD of DDP one sees dystrophy and destruction of epithelial cells, hyaline casts, and

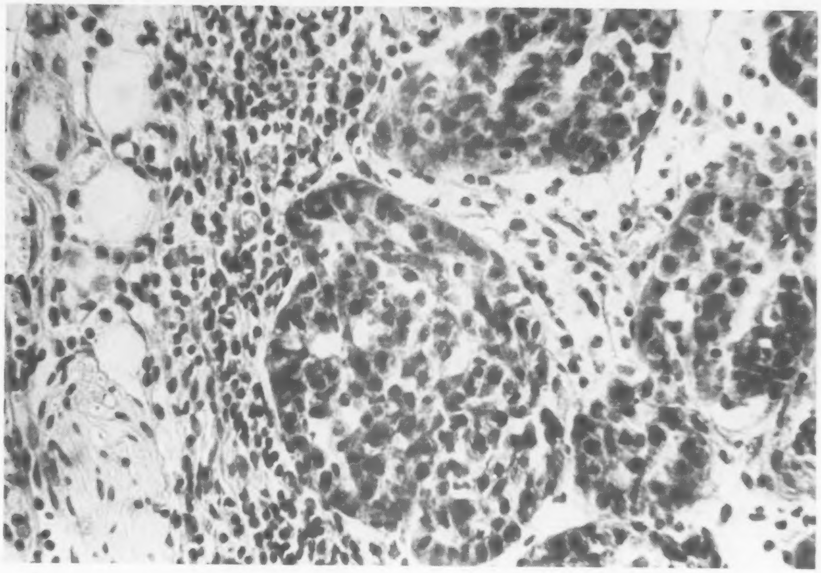

FIGURE 1.—Ca 755 3 days after a single injection of DDP (8.0 mg/kg). Note intense lymphohistiocytic reaction around the tumor (hematoxylin and eosin stain, × 200).

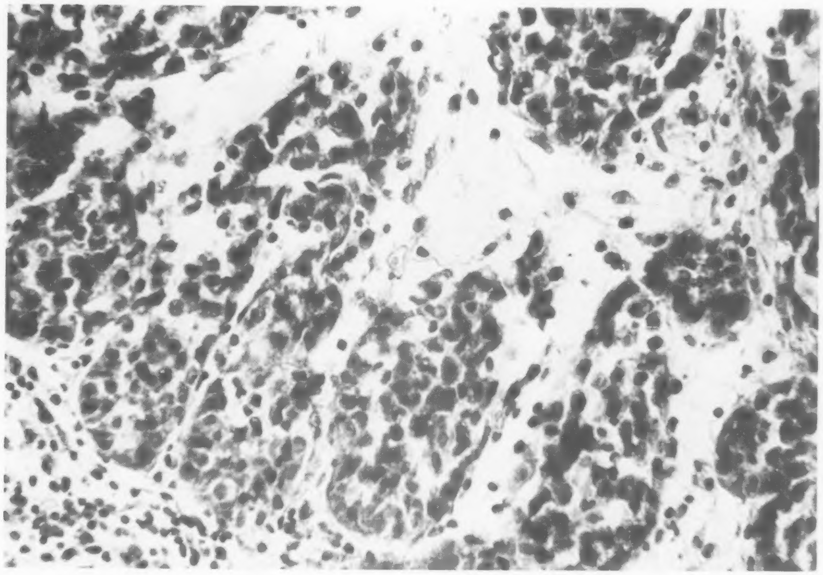

FIGURE 2.—Ca 755 5 days after a single injection of DDP (8.0 mg/kg). Note dystrophic changes and death of tumor cells (hematoxylin and eosin stain, × 200).

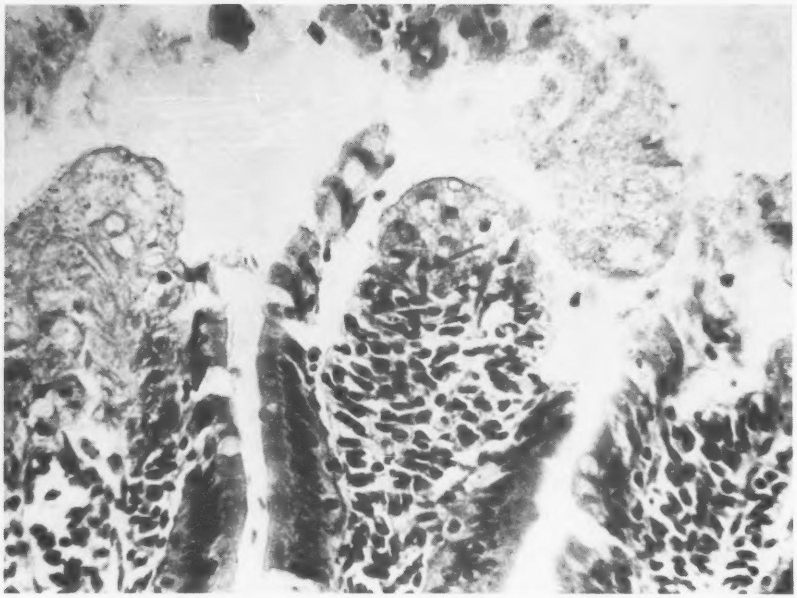

FIGURE 3.—Duodenum of mouse 24 hrs after a single injection of DDP (8.0 mg/kg). Epithelial cells of the apical villi are degenerating and peel off (hematoxylin and eosin stain, × 300).

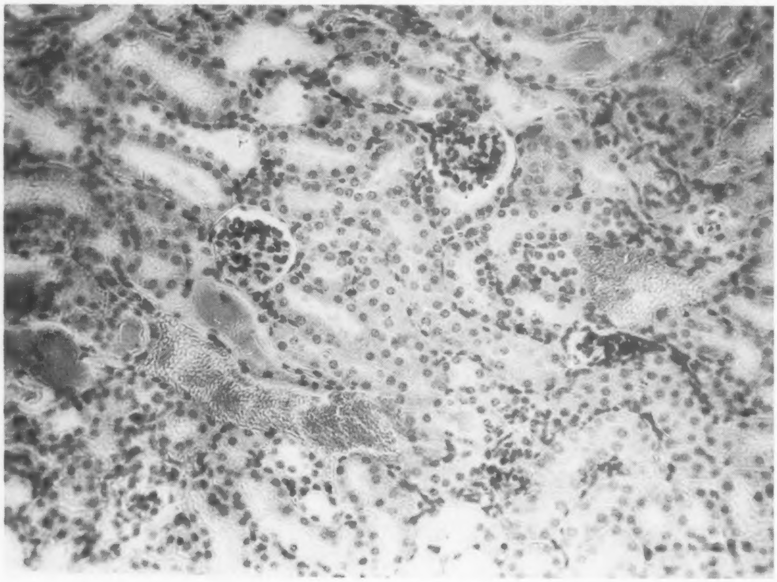

FIGURE 4.—Kidney of mouse 5 days after a single injection of DDP (8.0 mg/kg). Renal convoluted tubules show dystrophy of epithelial cells and appearance of hyaline casts. Note hemorrhagic focus (hematoxylin and eosin stain, × 100).

hemorrhagic foci in the renal convoluted tubules (fig 4). Sarcolysin given at the MTD does not damage the kidneys. On the fifth day after a single injection of DDP at the MTD, definite signs of hepatic lesions can be seen, which become worse by the ninth day. Tissue disintegrates, the parenchyma thins down, and the lobular structure of the organ is smoothed out and disappears. Vacuolization appears in the cytoplasm of the hepatic cells. Individual cells are destroyed by lysis. Sarcolysin given at the MTD does not damage the liver. DDP will also affect hemopoietic organs by causing bone marrow aplasia, splenic atrophy, and atrophy of thymus and lymph nodes. With tolerated doses of DDP these changes in normal tissues gradually disappear, but their regeneration takes quite some time. Fifteen days after injecting mice with a single MTD of DDP some organs still retain signs of damage.

It is necessary to stress an essential difference between the influence of DDP on tumor tissue and on the normal tissue. This concerns the difference in the speed of tissue regeneration after administration of the drug. In a number of normal organs, 15 days after injection of a single MTD of DDP, the reparative processes are still incomplete, while in Ca 755 in animals treated with the same dose of the drug, the regenerative process is practically terminated by the tenth day after treatment. By this time, damaged cells have disappeared from the tumor, the lymphohistiocytic reaction around and inside the tumor has disappeared, and the mitotic activity is again enhanced. Thus, the study shows that after DDP, regeneration in tumor tissue in mice is completed at least 5 days earlier than in some normal tissues.

DISCUSSION

This investigation has shown, above all, that the cytotoxic action of the platinum compound on tumor and normal cells differs basically from the cytotoxic effect produced by alkylating agents, in particular by sarcolysin, as demonstrated in this work and in our previous papers (3,15). One of the differences that separates the cytotoxic actions of sarcolysin and DDP on the tumor is the involvement in the latter case of immunocompetent cells of the host in the process of growth inhibition and regression of neoplasms; we believe that this powerful lymphohistiocytic reaction around and inside Ca 755 treated by DDP may be the result of an artificial heterogenization of tumor, a process intensely studied in the USSR by Svet-Moldavsky and Hamburg (16,17). The term "artificial heterogenization" proposed by these investigators implies the enhancement of tumor antigenicity by using different biologic agents (for instance, viruses) and chemical compounds. Artificial heterogenization of a neoplasm leads to an increase in its antigenic properties and hence stimulation of the host's reaction against tumor growth. The hypothesis of enhanced antigenicity of tumor as a possible mode of therapeutic action of DDP, advanced by Rosenberg (18), is confirmed by the present results as well as by a number of other reports.

It was noted that after complete cure of mice with solid Sarcoma 180 (5) and rats with Dunning ascitic leukemia (19), animals remained immune to repeated inoculations of these tumors for several months. The involvement of immune reactions of the host in the antitumor effect of DDP is clearly shown when combination therapy was given to mice with solid Sarcoma 180 to which was added a nonspecific immunostimulator (zymosan) and an immunodepressant (hydrocortisone). In the first instance, considerable enhancement of therapeutic effect of DDP was seen and in the second case its marked decrease could be noted (20).

In 1974, Sodhi and Aggarwal (21), by means of light and electron microscopy as well as by certain immunologic tests, studied special features of DDP action on solid Sarcoma 180. In contrast to Ca 755, the histologic changes of which (due to DDP therapy) had not been studied prior to our investigation, Sarcoma 180 regressed entirely after a single injection of the compound at the MTD (8 mg/kg) in the experiments of Sodhi and Aggarwal (21). Nevertheless, results of our experiments and those of Sodhi and Aggarwal were comparable in principle: in causing regression of Sarcoma 180 there was also a considerable increase in the number of lymphocytes, macrophages, and plasma cells. The method of double diffusion on agar revealed enhancement of antibodies against Sarcoma 180 treated with DDP. During the treatment period concentration of protein fractions α_1 and α_2 in serum of treated animals increased. Spleens of treated mice showed an increase in the number of lymphocytes and plasma cells. According to Sodhi and Aggarwal, these data point to an increased cell immunity of the host when DDP exerts an antitumor effect.

We believe that the investigation of the hypothesis of artificial heterogenization of tumors during chemotherapy with metallic coordination complexes has no theoretic significance but has practical significance. Our study of this problem is in progress and the results will be published later.

Many investigators have shown that DDP can be successfully used in combination with antitumor agents of other classes, namely, alkylating agents, antimetabolites, antibiotics, and plant alkaloids. This paper shows the efficacy of combination chemotherapy with DDP and sarcolysin (which has not been studied in combination with a platinum complex). Undoubtedly, the peculiarities of DDP's antineoplastic action, differences in side effects, and differences noted by us in the speed of regeneration of tumor and normal tissues under the influence of a platinum compound have definite bearing on the optimum schedules of the combined use of DDP with different chemotherapeutic agents. Considering the slow regeneration of affected normal tissues, optimum schedules of therapeutic use of DDP must apparently provide for considerable intervals between injections and courses of treatment. This point of view is based on our results: the most effective doses of DDP were large doses with considerable intervals between administrations. We have shown also that shortening the intervals between injections in combination cancer chemotherapy from 7 to 4 days markedly enhances toxicity of the drugs and leads to death in most of the treated mice.

Our experiments showed that the great variability of DDP's toxicity was due to different tumor and mouse strains. We believe that this very interesting and important observation requires special study. The above studies show that DDP is valid in chemotherapy for malignant tumors. Of particular interest is the beneficial effect of the use of DDP in combination with drugs possessing different modes of action. Maybe it will permit a decrease in the dose of the drug and thereby reduce its toxic effect on the host. The present study also points to the rationale of starting the synthesis and investigation of new metallic complexes of different metals which might possess greater antitumor activity and fewer side effects as compared with DDP. Such work is being conducted in many countries, including the USSR (22–24). We believe that in order to achieve further success in this direction of cancer chemotherapy, additional studies of biologic and antitumor peculiarities of DDP will be of great significance, as this drug will serve as a positive control in screening new metal complexes with antineoplastic activity.

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Effect of Actinomycin D and Neoarsphenamine on Tumor Control and Skin Tolerance in the Rat[1,2]

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SUMMARY

Actinomycin D and neoarsphenamine were tested for their ability to produce therapeutically favorable radiosensitization in the WAG/Rij rat. Acute and late skin reactions and control of the BA1112 rhabdomyosarcoma were examined in drug-treated and untreated animals irradiated in single- and five-fraction schedules. Actinomycin D was found to protect skin and tumors when added 15 minutes before irradiation. Actinomycin D added 2 hours before irradiation in a five-fraction trial produced slight tumor sensitization accompanied by slight skin protection. Neoarsphenamine produced significant tumor sensitization without skin sensitization in one of the single-fraction trials, but had no effect in the five-fraction trials.

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Current interest in radiosensitizers has concentrated on hypoxic cell sensitizers, such as metronidazole and misonidazole, which are believed to mimic the radiation sensitizing activity of oxygen. These electron-affinic compounds represent just one class of potentially useful radiosensitizers (1). This study reports tests of two drugs, actinomycin D and neoarsphenamine, which are thought to radiosensitize by other mechanisms.

Actinomycin D has been reported to radiosensitize mammalian cells in vitro (2), and to enhance radiation damage of normal tissues (3–8). Actinomycin D appears to sensitize by interfering with repair of radiation damage (2,6). The drug is used as a clinical cancer chemotherapeutic agent, usually in combination with other drugs and frequently as an adjunct to radiation therapy.

Neoarsphenamine, an organic trivalent arsenical compound, has been shown to radiosensitize bacteria, and in one bacterial species it preferentially sensitizes hypoxic cells (9,10). Neoarsphenamine may sensitize by binding naturally occurring sulfhydryl compounds which are radioprotectors (1,10).

Actinomycin D and neoarsphenamine were tested in combination with radiation for their effects on the tolerance of rat skin (11,12), and on the control of the BA1112 rhabdomyosarcoma (13,14). These systems have previously been used to study the therapeutic advantage of metronidazole (15), radioprotective compounds (16), and fractionation (14). This has the advantage of testing drug-radiation combinations in systems where there is already a detailed knowledge of the effects of radiation alone.

MATERIALS AND METHODS

Compounds.—Actinomycin D was obtained from Merck, Sharp and Dohme, Rahway, NJ. A stock solution was prepared in 100% ethanol and stored in the dark. For injection, this stock was diluted with a sterile 50% (vol:vol) glycerol:water solution. For measurements of tumor and blood levels of actinomycin D at various times after injection, tritiated actinomycin D (0.19 millicurie/g) was injected, and the actinomycin D was extracted and counted by the method of Schwartz et al (17). Neoarsphenamine was obtained from May and Barker, Ltd, Dagenham, England. The drug was stored under nitrogen and was dissolved in sterile saline (0.9 mg/ml NaCl) immediately before use. Concentra-

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tions of drug stock solutions were chosen so that all drug injections could be given ip at 0.2 ml/100 g of body weight.

Tumor system.—The tumor used was the BA1112 rhabdomyosarcoma, which is isologous in the WAG/Rij strain of rats. At a diameter of 5–10 mm the tumor has a volume doubling time of about 2 days and a hypoxic fraction of about 10% (13,18). Rats were inoculated in the subcutaneous tissue of the head, between the ears, with a suspension of tumor cells, and the tumors were treated when they reached a mean diameter of 7.5 mm. The rats were anesthetized with sodium pentobarbital 60 minutes before irradiation. Irradiation was done with a Siemens unit operated at 250 kV, 15 mA, with a half-value layer of 0.55 Cu (14). The dose rate to the midline of the tumor was 505 rads/minute, while the dose rate to the body ranged from 25 rads/minute cephalad to < 1 rad/minute caudad. The animals were checked for the presence of tumor every 10 days, and tumors were considered controlled if they had not regrown to 15 mm on the 120th day after treatment. This basis for calculating the 50% tumor control dose (TCD50) was necessary because recurrences continue to appear indefinitely and because scarring and necrosis prevent the prompt detection of many small recurrences; setting a later cutoff point for the definition of tumor control has only a small effect on the TCD50 (14).

Skin tolerance.—WAG/Rij rats were anesthetized with sodium pentobarbital 60 minutes before 250 kV irradiation of the left hind legs. Skin reactions were recorded twice weekly for 100 days using our standard scoring system (11). The conditions of irradiation were the same as those used for the tumor except that the dose rate to the skin of the leg was 590 rads/minute, while the dose rate to the body ranged from 20 rads/minute caudad to < 1 rad/minute cephalad. The doses required to produce skin damage in 50% of the animals treated (ED50) were calculated for five levels of reaction: acute partial moist desquamation, threshold acute necrosis, severe acute necrosis, threshold late skin damage, and severe late skin damage. These levels of skin damage were produced by single doses of 2250–4100 rads in control animals and allow analysis of drug effects over a range of doses.

Treatment regimens.—Each drug was tested in four drug-radiation regimens: a single injection of drug 15 minutes before a single dose of radiation; a single injection of drug 2 hours before a single dose of radiation; injections of drug 15 minutes before each of five irradiations (using a Mon-Wed-Fri-Mon-Wed treatment schedule); and injections of drug 2 hours before each of five irradiations (using a Mon-Wed-Fri-Mon-Wed treatment schedule).

Statistical methods.—A dose modification factor (DMF) was calculated for each tumor control and skin tolerance endpoint. The DMF for an endpoint is defined as the TCD50 or skin ED50 for the control animals divided by the equivalent TCD50 or skin ED50 for the drug-treated animals. A DMF of > 1.00 is an indication of sensitization, and a DMF of < 1.00 is an indication of protection. For each skin ED50 in a drug-radiation regimen, a therapeutic gain factor (TGF) was calculated. The TGF is defined as the DMF for tumor control divided by the DMF for skin tolerance. A TGF of > 1.00 indicates a therapeutically favorable result, with the tumor being sensitized more than the skin or the skin being protected more than the tumor. ED50 and TCD50 values were calculated by probit analysis according to the method of Finney (19). This method gives confidence limits that may not be symmetric on either linear or log scales. The standard errors quoted for TCD50 and ED50 values are the larger of the two 68% fiducial limits. The standard errors of DMF and TGF values were estimated to be the square root of the sum of the squares of the percent standard errors of the ED50 or TCD50 values. DMF and TGF values were considered to be significantly different from 1.00 if their values were two standard errors above or below 1. The validity of these assumptions about the significance of DMF values was tested by an alternative calculation. The nonparametric method of Kellerer and Brenot (20) provides a means for calculating confidence limits for DMF values that avoids probit analysis and the associated problems of calculating confidence limits for ratios of ED50 values. This method does not provide best-fit estimates of the DMF, and cannot be used to calculate TGF values or confidence limits for TGF values.

RESULTS

Control Animals

Control animals were irradiated along with the drug-treated animals in all schedules. In previous experiments, control animals were given ip injections of isotonic saline (15,16). In the experiments reported here, the control animals were given ip injections 15 minutes before irradiation using the actinomycin D carrier: 50% (vol:vol) glycerol:water. Tumor control doses, skin tolerance doses, and death rates were calculated for these animals and compared with values measured in uninjected and saline-treated animals. Table 1 shows that neither saline nor glycerol has a significant effect on tumor control or skin tolerance doses.

Table 1 also illustrates the accuracy with which TCD50 and skin ED50 values can be determined. For the relatively large numbers of animals treated with saline (40–50/endpoint), most of the standard errors are between 150 and 225 rads. For the glycerol-treated animals (30–35 animals/endpoint), the standard errors are larger. The 95% confidence limits for a comparison of the glycerol or saline animals to the nondrug animals are generally 0.87–1.15. The system, therefore, cannot detect sensitization at levels appreciably < 1.15

In the tumor control trials, the death rate in irradiated control animals has ranged from 12% to 18%. Most of these animals die from respiratory failure during or immediately after anesthesia. In the skin tolerance experiments, deaths in control animals are rare, but are also generally due to respiratory failure. For this reason, death rates in drug-radiation schedules must be corrected for the control death rates or the lethal effects of the drugs will be overestimated.

Actinomycin D

Actinomycin D is used in human cancer chemotherapy at daily doses of about 0.015 μg/g. Using the correction for body surface area proposed by Freireich et al (21), the equivalent rat dose would be 0.10 μg/g. This extrapolation is consistent with the reported 10% lethal dose (LD10) for daily ip injections of actinomycin D of 0.08–0.09 μg/g in rats (21). When we tested actinomycin D with irradiation in tumor animals we found lower LD10 values: 0.03 μg/g in five-fraction schedules and 0.09 μg/g in single-fraction schedules (table 2). The animals that died from actinomycin D doses near the LD10 died 7–14 days after treatment, and their appearance resembled that of animals dying from the bone marrow syndrome after whole-body irradiation.

Based on the results of the lethal-dose studies we tested actinomycin D at 0.10 μg/g in single-fraction trials and 0.025 μg/g in five-fraction trials. A single ip dose of 0.10 μg/g of actinomycin D gave serum

TABLE 1.—Tumor control and skin reaction doses for control animals

Endpoint	Animals receiving— No drug*	Saline†	Glycerol‡	All animals
		ED50 values for single doses§		
Tumor control	5538 ± 120	5222 ± 191	5683 ± 231	5457 ± 97
Acute, 1.75	2271 ± 80	2199 ± 92	2189 ± 164	2232 ± 59
Acute, 2.00	2715 ± 98	2741 ± 134	2915 ± 248	2739 ± 79
Acute, 2.25	3333 ± 126	3361 ± 187	3412 ± 274	3377 ± 98
Late, 0.75	2591 ± 126	2547 ± 195	2531 ± 212	2559 ± 95
Late, 1.75	4202 ± 109	3826 ± 169	4091 ± 249	4107 ± 89
		95% confidence limits on DMFs for single doses¶		
Tumor control		0.93–1.13	0.88–1.23	
Acute skin		0.93–1.25	0.81–1.25	
Late skin		0.94–1.19	0.84–1.21	
		ED50 values for 5 fractions in 9 days§		
Tumor control	6600 ± 159	6346 ± 248	6788 ± 548	6537 ± 118
Acute, 1.75	3517 ± 201	3655 ± 200	3568 ± 330	3564 ± 104
Acute, 2.00	3776 ± 100	4015 ± 162	4014 ± 229	3933 ± 82
Acute, 2.25	4328 ± 133	4305 ± 152	4441 ± 218	4345 ± 85
Late, 0.75	3827 ± 266	3644 ± 234	3443 ± 331	3661 ± 135
Late, 1.75	5386 ± 176	4988 ± 283	5225 ± 271	5164 ± 149
		95% confidence limits on DMFs for 5 fractions in 9 days¶		
Tumor control		0.87–1.19	0.81–1.10	
Acute skin		0.89–1.13	0.89–1.05	
Late skin		0.94–1.07	0.84–1.07	

*Tumor control data from ref 23; skin reaction data from refs 11 and 12.

†Animals injected with 0.018 mg/g of NaCl 15 mins before radiation (data from refs 15 and 16).

‡Animals injected with 2.0 μl/g of 50% (vol:vol) glycerol:water 15 mins before radiation.

§For tumor control, ED50 is the TCD50 with standard error; for skin reactions, ED50 is the dose producing a reaction equal to or greater than the specified reaction, with standard error.

¶95% confidence limits on the ratio of the ED50 for the saline- or glycerol-treated animals to the corresponding ED50 for the nondrug animals; calculated by the method of Kellerer and Brenot (20).

TABLE 2.—Lethal doses for actinomycin D and neoarsphenamine in irradiated tumor-bearing animals

Drug and schedule	LD10* (μg/g)	LD50* (μg/g)
Actinomycin D,† 1-fraction trial	0.09 (0.05–0.11)	0.17 (0.14–0.24)
Actinomycin D,‡ 5-fraction trial	0.03 (0.01–0.05)	0.14 (0.08–0.87)
Neoarsphenamine,† 1-fraction trial	40 (15–60)	135 (70–275)
Neoarsphenamine,‡ 5-fraction trial	25 (10–45)	95 (60–270)

*Calculated by probit analysis with correction for death rate in irradiated control animals; shown with 95% confidence intervals.

†Drug given 15 mins before a 6250-rad irradiation of the tumor.

‡Drug given 15 mins before each 1250-rad irradiation of the tumor using a 5-fraction, 9-day treatment schedule.

concentrations of 0.013–0.016 μg/ml at 15 minutes, which fell to 0.004–0.005 μg/ml at 2 hours. Tumor concentrations of actinomycin D were 0.002–0.004 μg/g at 15 minutes and rose to 0.007–0.011 μg/g at 2 hours.

Twenty-nine to 33 animals were treated in each of the tumor control trials and 24–27 animals were treated in each of the skin tolerance trials. Death rates are higher in tumor animals treated with actinomycin D 15 minutes before irradiation than in animals treated with actinomycin D 2 hours before irradiation. However, when the death rates for either tumor or skin animals are compared with the controls, the differences are not significant.

For the purpose of evaluating the effect of actinomycin D, DMFs were calculated for skin tolerance and tumor control in the four actinomycin D treatment regimens. At the top of table 3 are DMF values based on TCD50 and skin ED50 values determined by probit analysis. The severe late skin reaction endpoint could not be evaluated in the five-fraction schedules because of a lack of treatments at high doses. The bottom portion of table 3 shows 95% confidence limits for the DMF values based on the nonparametric procedure of Kellerer and Brenot (20).

When actinomycin D was added 2 hours before treatment, no significant sensitization of skin or tumors was seen in either the single- or the five-fraction trials. For threshold acute necrosis, probit analysis shows slight skin protection for both single and five fractions. For actinomycin D given 2 hours before treatment, neither sensitization nor protection is evident from the nonparametric analysis.

Protection is significant when actinomycin D is given 15 minutes before treatment. It is seen for most levels of acute skin damage in both single- and five-fraction trials, and this is reflected in the nonparametric analysis of acute skin damage. This protection is not seen for late skin damage. Actinomycin D added 15 minutes before treatment had no effect on tumor control in the single-dose schedule but showed significant protection in the five-fraction schedule.

Neoarsphenamine

As an antisyphilitic, neoarsphenamine was used in man at weekly doses of 5–10 μg/g (22). Using the correction for surface area (21), the equivalent rat dose would be 35–70 μg/g. When we tested neoarsphenamine in irradiated tumor animals we found an LD10 of 40 μg/g in single-fraction trials and 25 μg/g in five-fraction trials (table 2). Based on this we tested neoarsphenamine at 50 μg/g in single-fraction schedules and at 25 μg/g in five-fraction schedules.

Thirty-four to 37 animals were used in the neoarsphenamine tumor control trials and 25–29 animals were used in each of the skin tolerance trials. In neoarsphenamine trials, the death rates among drug-treated animals were not significantly different from those of the controls. Table 4 shows the DMF values in the four neoarsphenamine regimens. The severe acute necrosis and severe late reaction endpoints could not be evaluated in the two five-fraction regimens because of a lack of treatments at high doses.

Neoarsphenamine had no significant effect on acute skin damage in either the single- or five-fraction regimens. For late skin damage there is some indication of sensitization. This is seen in the significantly high DMF for a single dose of neoarsphenamine given 15 minutes before treatment, and in the asymmetry of the confidence limits for late damage in all regimens.

Significant tumor sensitization was seen when neoarsphenamine was given 2 hours before a single dose of radiation. There was a suggestion of tumor sensitization when neoarsphenamine was given 15

TABLE 3.—DMFs for actinomycin D

Endpoint	Drug-radiation schedule			
	1 fraction, drug at 15 mins	1 fraction, drug at 2 hrs	5 fractions, drug at 15 mins	5 fractions, drug at 2 hrs
*DMF values based on probit analysis**				
Tumor control	0.95 ± 0.05	0.99 ± 0.05	0.86 ± 0.03†	1.03 ± 0.03
Acute partial moist desquamation	0.99 ± 0.15	1.08 ± 0.15	0.90 ± 0.05†	1.01 ± 0.06
Threshold acute skin necrosis	0.81 ± 0.05†	0.89 ± 0.05†	0.89 ± 0.03†	0.92 ± 0.04†
Severe acute skin necrosis	0.83 ± 0.07†	0.95 ± 0.06	0.88 ± 0.05†	0.96 ± 0.04
Threshold late skin damage	1.02 ± 0.15	1.08 ± 0.14	0.85 ± 0.08	0.92 ± 0.06
Severe late skin damage	0.92 ± 0.09	0.94 ± 0.09		
95% confidence limits on DMFs‡				
Tumor control	0.79–1.04	0.85–1.13	0.67–0.96†	0.87–1.15
Acute skin damage	0.81–1.00†	0.81–1.05	0.83–0.97†	0.88–1.12
Late skin damage	0.82–1.13	0.89–1.28	0.69–1.07	0.74–1.07

*DMF for tumor control = (TCD50 for controls)/(TCD50 for drug-treated animals) and DMF for a skin reaction = (skin ED50 for controls)/(skin ED50 for drug-treated animals), with standard error.

†DMF significantly < 1.00 ($P < 0.05$).

‡95% confidence limits calculated by the nonparametric method of Kellerer and Brenot (20).

TABLE 4.—DMFs for neoarsphenamine

Endpoint	Drug-radiation schedule			
	1 fraction, drug at 15 mins	1 fraction, drug at 2 hrs	5 fractions, drug at 15 mins	5 fractions, drug at 2 hrs
*DMF values based on probit analysis**				
Tumor control	1.06 ± 0.04	1.13 ± 0.04†	1.01 ± 0.03	1.01 ± 0.03
Acute partial moist desquamation	1.07 ± 0.08	0.90 ± 0.08	0.99 ± 0.07	0.94 ± 0.06
Threshold acute skin necrosis	0.99 ± 0.07	1.04 ± 0.11	0.91 ± 0.06	0.95 ± 0.07
Severe acute skin necrosis	0.97 ± 0.06	0.98 ± 0.08		
Threshold late skin damage	1.19 ± 0.20	1.05 ± 0.11	1.08 ± 0.15	1.02 ± 0.09
Severe late skin damage	1.18 ± 0.08†	1.03 ± 0.11		
95% confidence limits on DMFs‡				
Tumor control	0.96–1.24	1.03–1.24†	0.95–1.14	0.87–1.14
Acute skin damage	0.88–1.16	0.88–1.10	0.87–1.13	0.87–1.13
Late skin damage	0.92–1.39	0.94–1.19	0.91–1.31	0.91–1.31

*DMF for tumor control = (TCD50 for controls)/(TCD50 for drug-treated animals) and DMF for a skin reaction = (skin ED50 for controls)/(skin ED50 for drug-treated animals), with standard error.

†DMF significantly > 1.00 ($P < 0.05$).

‡95% confidence limits calculated by the nonparametric method of Kellerer and Brenot (20).

minutes before a single dose, but neither probit nor nonparametric analysis of the data shows significance at the 95% level. In the five-fraction regimens neoarsphenamine had no effect on tumor control.

DISCUSSION

The critical question in these experiments is whether the drugs can alter the relative sensitivities of the tumor and normal tissue to produce more tumor destruction with less normal tissue damage. To answer this question, a TGF was calculated for each of the eight drug-radiation regimens. Because the DMF values for skin were found to depend on the level of reaction, TGFs have been calculated for all five levels of acute and late skin damage. These TGF values are shown in table 5, with the mean of the TGF values for each regimen.

In only two schedules are the TGF values significantly and consistently > 1: when a single dose of neoarsphenamine is given 2 hours before irradiation (mean TGF, 1.14), and when actinomycin D is given 2 hours before irradiation in a five-fraction schedule (mean TGF, 1.08). These should be compared with the mean TGF of 1.15 in the best metronidazole regimen (15) and the mean TGF of 1.26 in the best protector regimen (16).

In the successful neoarsphenamine trial, the favorable TGF is due to significant tumor sensitization in the absence of significant skin sensitization. However, the tumor sensitization is less than 1.23–1.26 achieved for BA1112 with metronidazole (15). Like neoarsphenamine, metronidazole does not sensitize BA1112 in five-fraction trials. It is thought that the BA1112, although initially hypoxic (13), may reoxygenate well in a five-fraction schedule (14,23,24). Thus, sensitization of BA1112 in a single-fraction trial but not in a five-fraction trial may be an indication of differential sensitization of hypoxic cells.

The actinomycin D trials are difficult to interpret because of the unexpected skin protection. When actinomycin D is added 2 hours before treatment, the protective effect is slight and may not be significant. However, when actinomycin D is added 15 minutes before treatment, the protection is significant for five of the nine skin tolerance endpoints. This is in contrast to the skin sensitization by actinomycin D reported by D'Angio et al (3).

There is one previous report of a similar phenomenon in animals. Griem and Ranniger (4) studied the effects of actinomycin D and radiation on hair dysplasia in mice. They found that 0.10 μg/g of actinomycin D added 2–16 hours before irradiation caused sensitization. However, 0.15 μg/g at 1, 12, or 36 hours before irradiation produced significant protection.

There is no definite explanation for this anomalous protective effect seen with actinomycin D. Anesthetized animals given actinomycin D in these trials show an immediate, but temporary, loss of color and a drop in body temperature. This suggests that the high dose of actinomycin D may cause a shock reaction resulting in slight whole-body hy-

TABLE 5.—TGFs for neoarsphenamine and actinomycin D*

Drug	Tissue endpoint					
	Acute partial moist desquamation	Threshold acute skin necrosis	Severe acute skin necrosis	Threshold late skin damage	Severe late skin damage	Mean TGF for regimen
Neoarsphenamine						
1 fraction, drug at 15 mins	0.99 ± 0.09	1.07 ± 0.09	1.09 ± 0.08	0.89 ± 0.15	0.90 ± 0.08	0.99 ± 0.08
1 fraction, drug at 2 hrs	1.26 ± 0.11†	1.09 ± 0.13	1.15 ± 0.10	1.08 ± 0.12	1.10 ± 0.12	1.14 ± 0.08‡
5 fractions, drug at 15 mins	1.02 ± 0.08	1.11 ± 0.08		0.94 ± 0.07		1.02 ± 0.07
5 fractions, drug at 2 hrs	1.07 ± 0.07	1.06 ± 0.08		0.99 ± 0.09		1.04 ± 0.04
Actinomycin D						
1 fraction, drug at 15 mins	0.96 ± 0.15	1.17 ± 0.10	1.14 ± 0.10	0.93 ± 0.14	1.03 ± 0.11	1.05 ± 0.10
1 fraction, drug at 2 hrs	0.92 ± 0.15	1.11 ± 0.08	1.04 ± 0.07	0.92 ± 0.14	1.05 ± 0.11	1.01 ± 0.08
5 fractions, drug at 15 mins	0.96 ± 0.06	0.96 ± 0.04	0.98 ± 0.06	1.01 ± 0.09		0.97 ± 0.02
5 fractions, drug at 2 hrs	1.02 ± 0.07	1.12 ± 0.06†	1.07 ± 0.05	1.12 ± 0.08		1.08 ± 0.04‡

*TGFs are defined as the ratio of the DMF for tumor control and the DMF for the skin tolerance endpoint, using data from tables 3 and 4.

†TGF significantly > 1.00 ($P < 0.05$).

‡TGF for all skin endpoints, as a group, significantly > 1.00 ($P < 0.05$).

poxia or hypothermia. Both of the latter effects would be expected to protect both tumor and normal tissue.

The failure to achieve tumor sensitization with actinomycin D may be a result of the insensitivity of this tumor to actinomycin D, but it may also be that the tumor sensitization is obscured by the nonspecific protection. This may be the case in the five-fraction trial where actinomycin D was given 2 hours before each irradiation. The favorable TFG in that trial is due to slight tumor sensitization combined with slight skin protection.

The considerable difficulties involved in determining whether a specific drug is of potential use in radiotherapy are illustrated by the fact that both actinomycin D and neoarsphenamine showed therapeutically favorable results in only one of four regimens tested. Nevertheless, these studies do show that drugs other than the electron-affinic hypoxic cell sensitizers are capable of sensitizing tumors more than normal tissues under certain conditions. The results achieved with neoarsphenamine and actinomycin D are, however, less favorable than those achieved with metronidazole or radioprotectors in this system.

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Antimetastatic Action of Some Triazene Derivatives Against the Lewis Lung Carcinoma in Mice[1]

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SUMMARY

Two dimethyltriazenoimidazoles, DTIC and BRL 51308, and a benzenoid dimethyltriazene, CB 10286, have been examined for their effects in mice bearing Lewis lung carcinoma. A slight reduction of primary tumor growth was found after treatment with DTIC and BRL 51308, whereas CB 10286 caused no significant effect. On the contrary, all the tested compounds sharply reduced the number of lung metastases and also resulted in a high proportion of animals free of metastases at death. No significant cytotoxic effect of the triazenes was observed in small established pulmonary tumors, as determined by evaluating the effects of treatment on the fractional incorporation of ^{3}H-TdR into DNA of the lung colonies. These results are in contrast to those obtained with a purely cytotoxic agent, cyclophosphamide, and indicate that all three triazene derivatives tested have selective antimetastatic properties.

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The derivatives of dimethyltriazene form a class of compounds with interesting antineoplastic properties (1–9) (fig 1). The imidazole carboxamide derivative DTIC exhibits a broad spectrum of activity against transplantable rodent tumors (1), and is effective against human melanoma either as a single agent (2) or in combination with immunostimulants (3). One analog of DTIC, BRL 51308, has been reported to possess antimetastatic activity in mice bearing the Lewis lung carcinoma (10). No other triazene derivative appears to have been examined for selective antimetastatic activity. It seemed desirable, therefore, to compare the antimetastatic effects of BRL 51308 with those of DTIC and a benzenoid derivative, CB 10286, in order to determine if the antimetastatic properties of BRL 51308 were peculiar to this compound or if they might represent a general property of the dimethyltriazene moiety. The effects of these three compounds on primary tumor growth and the development of metastases have been examined in mice bearing the Lewis lung carcinoma. In order to gain more information on the mechanism of the antimetastatic action, the effects of treatment on the fractional incorporation of ^{3}H-TdR into DNA (11) have been studied in the primary tumor and in the metastases.

MATERIALS AND METHODS

Lewis lung carcinoma.—Fragments, approximately 1 mm^3, were prepared aseptically from donor mice inoculated with tumor 2 weeks previously, and were implanted sc under light ether anesthesia into the axillary region of C57BL mice. Primary tumor growth was assessed by caliper measurements and the tumor volume was calculated from the expression $V = (L \times W^2)/2$ where L and W are the longest and shortest diameters respectively (12). At death, the lungs were removed and the number of macroscopically detectable metastases was counted using a dissection magnifying lens.

Cytotoxic drugs.—The DTIC used in these studies was kindly supplied by Dr. H. B. Wood, Jr, National Cancer Institute, Bethesda, Md, and BRL 51308 was a generous gift from Dr. J. Heyes, Beecham Pharmaceuticals Ltd, Harlow, Essex, England. CB

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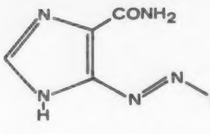
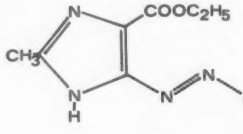
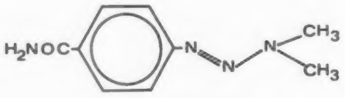


5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide



4-carbethoxy-5-(3,3-dimethyl-1-triazeno)-2-methylimidazole



1-p-carboxamidophenyl-3,3-dimethyltriazene

FIGURE 1.—Chemical structure of the triazene derivatives tested.

TABLE 1.—Ip toxicity of the tested compounds in mice

	LD50*	
Compound	Repeated administration† (mg/kg/day)	Single administration (mg/kg)
DTIC	112 (178–70)	567 (692–465)
BRL 51308	112 (178–70)	567 (692–465)
CB 10286	56 (89–35)	356 (566–224)

*LD50 values have been obtained using groups of 3 non-tumor-bearing C57BL mice by the method of Spearman-Kärber (22). Nos. in parentheses = confidence limits (P = 0.05).

†Daily injections for 14 subsequent days.

10286 was synthesized by previously described methods (13,14). All the compounds were suspended by sonication in arachis oil-acetone (9:1, vol/vol) and administered by ip injection of 0.05 cm^3/10 g of body weight.

Measurement of fractional incorporation.—The fractional incorporation of ^{3}H-TdR into DNA (^{3}H in DNA at 1 hour/total ^{3}H in the tissue) was determined by the methods described by Houghton and Taylor (11).

RESULTS

The effects on the growth of the primary Lewis lung carcinoma of 14 consecutive daily doses of each of the three drugs (beginning on the day following tumor implantation) are illustrated in figure 2. For each of the three compounds studied, the highest dose administered was equitoxic and equal to one half of the LD50 determined for C57BL mice using the same treatment schedule (table 1). All the tumors were measured 15 days after tumor implantation. The most pronounced reduction in tumor volume, to about 60% of that observed in untreated controls, occurred with DTIC and the effect was of a similar magnitude for each of the three dose levels examined. No significant effect on tumor growth was observed with CB 10286, and a decrease in tumor volume was found only at the two higher dose levels with BRL 51308.

The number of lung metastases observed in the lungs of the mice killed at 22 days after tumor implantation is listed in table 2. Each of the three triazene derivatives studied caused a marked, dose-dependent reduction in the number of macroscopically detectable lung colonies. There were similar reductions in both the numbers of large and small lung nodules. At the highest dose level tested, the treatment with each drug resulted in a high proportion of animals with no detectable pulmonary metastases. This was especially marked with CB 10286 where no nodules were observed in any of the eight mice in the group.

The effects of single equitoxic doses of each of the three drugs at one half the LD50 for single doses (table 1) on the fractional incorporation of TdR into subcutaneous and pulmonary Lewis lung tumors and into normal mouse bone marrow and intestinal mucosa at 24 and 48 hours after drug administration to animals bearing 16-day-old tumors are listed in table 3. Only four animals were used in each group and the data are shown as percentages of the fractional incorporation observed in the tumors and tissues of untreated tumor-bearing control mice.

DISCUSSION

Treatment of mice bearing Lewis lung tumors with any of the three triazene derivatives tested resulted in only a slight delay in the growth of the primary tumor. The order of response was DTIC > BRL 51308 > CB 10286, with the last compound producing no significant delay in tumor growth. In contrast, all three compounds produced marked

TABLE 2.—Effects of the tested compounds on the number of lung metastases*

Compound	Daily dose (mg/kg)	Average No. of metastases/ mouse (± SE)		Animals free of metastases/ total treated
		Small†	Large	
Control	—	18.73 ± 2.97	7.82 ± 1.75	0/8
DTIC	15	5.00 ± 0.82	3	3/8
	30	2.25 ± 1.44	1.67 ± 0.82	2/8
	60	1	1	6/8
BRL 51308	15	11.00 ± 8.03	1	0/8
	30	2.00 ± 0.50	1	1/8
	60	5	0	7/8
CB 10286	7.5	15.33 ± 3.27	3.50 ± 1.67	0/8
	15	6.00 ± 1.57	1.67 ± 0.41	1/8
	30	0	0	8/8

*Treated animals, whose primary tumor growth is reported in fig 2, were killed on Day 22 after tumor inoculation, and the macroscopically detectable lung metastases were counted.

†Diameter < 2 mm.

reductions in the number of pulmonary metastases observed in the treated animals. At the lowest dose level, one eighth of the LD50, DTIC was more effective in suppressing the formation of pulmonary nodules than BRL 51308 or CB 10286. However, at the highest dose level tested, no pulmonary metastases were found in any of the animals treated with CB 10286.

The lack of correlation between inhibition of primary tumor growth and depression of metastasis formation observed for the tested compounds is similar to that reported for ICRF-159 (15,16), and indicates that all three of the triazene derivatives tested possess some selective antimetastatic properties.

The purpose of the limited studies of the effects of these drugs on the fractional incorporation of TdR into pulmonary and subcutaneous tumors was to examine whether the antimetastatic effects of these agents were due to a selective effect on cell proliferation in metastatic tumors. The results presented in table 3 show that none of the three drugs tested show any significant effect on the level of DNA synthesis in pulmonary metastases. Both CB 10286 and DTIC cause small reductions in fractional incorporation into primary tumors at 24 hours after drug administration, but these effects are transient and control levels of incorporation have been regained by 48 hours. All three drugs showed a transient depression of DNA synthesis in the intestinal mucosa. These data are in marked contrast to the effects of cyclophosphamide on pulmonary and subcutaneous Lewis lung tumors, where parallel inhibition of growth (17) and marked depression of fractional incorporation are observed in both tumor locations (11).

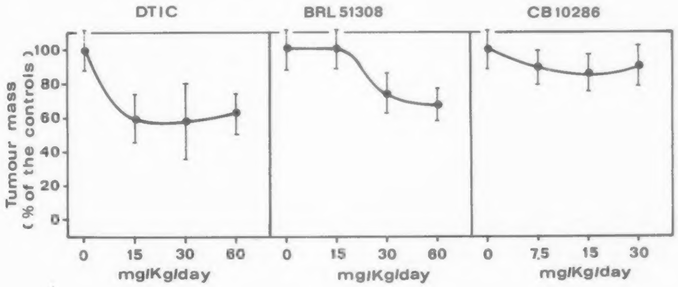


FIGURE 2.—Effects of the tested compounds on primary tumor growth. Treatment was performed daily, from Days 1 to 14 after tumor inoculation; the evaluation of tumor mass was performed on Day 15. Each value is the mean (± SE) obtained using groups of 8 mice.

TABLE 3.—Effects of the tested compounds on the fractional incorporation of ^{3}H-TdR into subcutaneous and pulmonary Lewis lung tumors and normal mouse bone marrow and intestinal mucosa

Tissue	Drug/ dose level (mg/kg)	Fractional incorporation (% of controls)	
		24 hrs	48 hrs
Subcutaneous tumor	BRL 51308/285	100 ± 2	101
Pulmonary tumor		95 ± 8	84 ± 10
Intestinal mucosa		55 ± 6	100
Bone marrow		98	58 ± 10
Subcutaneous tumor	CB 10286/168	64 ± 7	97 ± 7
Pulmonary tumor		99 ± 4	91 ± 5
Intestinal mucosa		53 ± 2	99 ± 2
Bone marrow		98	109
Subcutaneous tumor	DTIC/285	73 ± 11	97 ± 9
Pulmonary tumor		94 ± 7	95 ± 10
Intestinal mucosa		47 ± 14	103 ± 2
Bone marrow		101	104

The antimetastatic effects of these triazene derivatives do not, therefore, appear to result from the killing of cells in small established metastases. Thus, the effect must involve other mechanisms such as interference with the metabolism of single cells, or their implantation and multiplication in the lungs. If, as has been suggested (18), proteolytic enzymes of tumor origin play a role in tumor dissemination, it is interesting to note that CB 10286 has been shown to inhibit neutral proteases in vitro (19), and that neutral protease inhibitors reduced metastasis formation in mice bearing Lewis lung carcinoma (20,21).

Further studies designed to clarify the mechanism of the antimetastatic action of triazenes and to determine the nature of any structure-antimetastatic activity relationships for the benzenoid derivatives are in progress.

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Therapeutic Implications of Cell Kinetic Changes After Cyclophosphamide Treatment in "Spontaneous" and "Transplantable" Mammary Tumors[1,2,3]

Paul G. Braunschweiger* and Lewis M. Schiffer[4,5]

SUMMARY

The present studies were initiated to investigate the cell kinetics in spontaneous and transplantable mammary tumors after single doses of cyclophosphamide (Cp). The ^{3}H-thymidine (^{3}H-TdR) labeling index (LI), the DNA synthesis times, and the primer-dependent DNA polymerase labeling index (PDPI) were determined by in vitro methods for spontaneous mammary tumors (SMT) in C3H/HeJ retired breeders and 13762 transplantable rat mammary tumors (RMT) in Fischer 344 rats from 6 hours to 14 days after Cp treatment. The perturbations in the cell kinetics, although quantitatively different in the two tumor models, were qualitatively similar in that transient changes in both PDPI and ^{3}H-TdR LI were observed within the first 24 hours after treatment. These changes were followed by a variable period during which cell proliferation was suppressed. Increases in the PDPI and ^{3}H-TdR LI, presumably reflecting proliferative recovery, were observed prior to the initiation of tumor regrowth. Increases in the ^{3}H-TdR LI and PDPI within the first 24 hours after Cp in C3H/HeJ SMT indicated an interval of increased sensitivity to adriamycin. In the 13762 RMT, combination chemotherapy protocols designed to exploit changes in cell kinetics at early times (24 hours) and during the recovery phase of the response resulted in greater long-term tumor-free survival.

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Cyclophosphamide (Cp) is one of the most widely used and, in many cases, one of the most effective antitumor agents (1). Although it is useful alone in many experimental tumor systems, it is often more effective in combination with other drugs (2–11) and/or surgery (12,13). Clinically, Cp has been shown to have activity against a wide range of human tumors when used singly (14,15) or in combination (15–17).

The exact mechanism of action of Cp is not completely understood. It is known, however, that the drug is rapidly converted to aldophosphoramide and other metabolites in the liver which may act as nonspecific alkylators that primarily affect proliferating cells (18). Studies with experimental solid tumor models have shown that this agent is more effective in small tumors (8,12,13) than in large tumors, and that it is more effective in tumors with rapid doubling times (14). Studies with experimental and human solid tumors have shown the drug to be more effective when administered in large intermittent doses than when administered daily (4,19,20). Many investigators have suggested that Cp can be effectively employed as a priming agent to reduce the tumor burden and thus create a more highly proliferative cell population that may be more susceptible to subsequent administration of cycle stage specific agents (1,2,7,8,12,13,15–17). It has not yet been fully appreciated that the timing of the proliferative response following a single Cp dose could dictate the appropriate timing for subsequent treatment. Since cell kinetic determinations in the per-

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[3] Cyclophosphamide was supplied by Mead Johnson Laboratories, Evansville, Ind.

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turbed state cannot be accurately measured by the percent labeled mitoses method, few studies have been undertaken to examine the cell kinetics following perturbations with cytotoxic agents.

Recently, we reported methods by which cell kinetics can be rapidly and reliably determined for solid tumors in vitro. These methods include determination of the ^{3}H-thymidine (^{3}H-TdR) labeling index (LI), the DNA synthesis time (T_S), and the primer-dependent DNA polymerase labeling index (PDPI), an estimate of tumor growth fraction (21,22). The present studies were initiated to determine if treatment with single doses of Cp could produce changes in the cell kinetics of C3H/HeJ spontaneous mammary tumors (SMT) and 13762 transplantable rat mammary tumors (RMT), and, further, what implications these changes might have for subsequent therapy.

MATERIALS AND METHODS

Animals.—Nearly 1000 JAX C3H/HeJ retired breeders are maintained in our animal facilities and checked weekly for the appearance of newly developed SMT. Fischer 344 rats, approximately 35 days old, were used to maintain the 13762 mammary tumor line. All animals were maintained on a 12-hour light-dark cycle and fed Purina laboratory chow and water ad libitum.

Mammary tumor models.—Tumor-bearing C3H/HeJ retired breeders were culled weekly. Tumors were measured by caliper in three axes, and tumor volumes were calculated (21). Measurements were made at least twice weekly for at least 2 weeks prior to study. The 13762 RMT, originally induced with 7,12-dimethylbenzanthracene by Segaloff (23), was obtained from Dr. Arthur Bogden (Mason Research Institute, Worcester, Mass). It has been routinely maintained in this laboratory by sc implantations of 2–3-mm^3 pieces in young adult Fischer 344 rats with 100% tumor takes. Tumor sizes were determined by caliper measurements in two dimensions $\left(\frac{l + w}{2}\right)$ (24) starting on Day 7 after implantation.

Drug treatments.—Tumor-bearing C3H/HeJ mice were weighed and given 170 or 300 mg/kg of Cp in sterile saline by ip injection when tumor volumes were approximately 1 cm^3. Rats bearing the 13762 RMT were weighed and given 80 mg/kg of Cp on Day 14 after transplantation when tumors were 2.5–3 cm in diameter. Tumor-bearing animals were killed at various times after treatment for in vitro cell kinetic studies. At least four animals were used at each study interval from 6 hours to 14 days after treatment. Adriamycin (supplied by Adria Laboratories, Inc, Wilmington, Del) in sterile saline was also administered by ip injection. All drugs were made up immediately prior to use.

Cell kinetic measurements.—The methods for in vitro single and double labeling of solid tumors have been previously described in detail (21). Briefly, tumor-bearing animals were killed by cervical dislocation. The tumors were resected and minced in McCoy's media supplemented with fetal calf serum (Grand Island Biological Co, Grand Island, NY). The tumor cell suspensions were then either single-labeled with ^{3}H-TdR (15–17 curies/mM, 3 microcuries/ml) for 1 hour or double-labeled, for the determination of T_S, with ^{3}H-TdR as above, and then for an additional 30 minutes with ^{14}C-TdR. All in vitro labeling was done at 37°C in a shaking water bath. Following the labeling procedure, viable cells were separated by Ficoll-Hypaque gradient centrifugation and slide preparations were made for autoradiography as previously described (21,25).

The primer-dependent DNA polymerase (PDP) assay is an autoradiographic method to measure the simultaneous presence of nuclear DNA polymerase and nuclear DNA primer template activity in individual cell nuclei. This technique has also been previously described (22,25–27). Briefly, imprints from a freshly cut tumor surface were made on microscope slides and air-dried. The cytoplasm was stripped from the cells by dipping in a 0.25% agar solution at 40°C and air-dried. This process serves to remove the cytoplasm, leaving only bare nuclei adherent to the slide. The slides were then fitted with a glass incubation chamber into which 0.5 ml of an incubation mixture containing the deoxy-5′-triphosphates of adenosine, guanosine, and cytidine, 5 mM $MgCl_2$, Ficoll (Sigma Chemical Co, St. Louis, Mo), and 5 microcuries of ^{3}H-TdR triphosphate (specific activity, 54 curies/mM, Schwarz/Mann) in 0.02 M tris buffer (pH 7.4) was added. The preparations were incubated at 37°C for 45 minutes at which time the reaction was stopped on ice. The incubation chambers were then removed, the slides rinsed, and the cells fixed in acidic formaldehyde and rinsed again prior to subsequent autoradiographic analysis.

Autoradiography.—Kodak NTB-2 liquid photographic emulsion was used throughout. The emulsion was applied by dipping at 45°C, and the slides were exposed for from 1 to 7 days in the dark at 4°C with drierite. The exposed autoradiograms were developed by gold activation autoradiography previously reported by us (21,25) to reduce

TABLE 1.—Unperturbed cell kinetics in C3H/HeJ and 13762 mammary tumors*

Tumor	LI (%)	T_S (hrs)	PDPI (%)	T_P (hrs)	T_C (hrs)	λ	T_d (days)
C3H/HeJ†	7.4	10.9	16.6	134.7	28.8	0.72	
± 1 SEM	0.5	0.2	0.4	7.4	1.6		12.4
13762 (14-day)	31.8	5.6	69.8	15.5	11.9	0.89	4.5
± 1 SEM	2.1	0.2	2.0	0.8	0.6		

*T_P = potential doubling time; λ = correction for nonlinear S phase distribution (ref 29). All other abbreviations are explained in the text.

†Previously reported (ref 25).

the autoradiographic exposure times required for adequate grain densities. Single-label (^{3}H-TdR) samples and PDP samples were exposed for 1 and 7 days, respectively, while for the double-label samples, double emulsion autoradiography was employed (21). The ^{3}H-TdR LIs and PDPIs were determined by examining at least 500 cells while the T_S was determined by examining at least 300 labeled cells in double emulsion autoradiograms.

Calculations.—The ^{3}H-TdR LI and the PDPI are direct visual measurements of labeled fractions of a population. The T_S was calculated from:

$$T_S = \frac{\text{Ta} \times {}^{14}\text{C labeled cells}}{{}^{3}\text{H only labeled cells}} \qquad [1]$$

where Ta is the interval between administration of the labeled thymidines (28). Cell cycle parameters were calculated as previously described (25). The t test was used for statistical analysis of the data.

RESULTS

Table 1 shows the unperturbed cell kinetics, determined in vitro, for the two tumor systems used in the present studies. The C3H/HeJ SMT is a slowly proliferating, low growth fraction tumor with a cell cycle time (T_C) of approximately 29 hours. In contrast, the 13762 RMT is a rapidly proliferating, high growth fraction tumor with a T_C of approximately 12 hours. In vitro estimates of cell kinetic parameters were similar to values obtained in vivo for both the C3H/HeJ SMT (25) and the 13762 RMT (unpublished percent labeled mitoses data).

Figure 1 shows the growth of C3H/HeJ SMT and 13762 RMT after single doses of Cp. The growth data have been normalized to treatment day tumor size for ease of comparison. Pretreatment tumor values were approximately 1.0 and 6.7 cm^3, respectively, for C3H/HeJ SMT and 13762 RMT. C3H/HeJ SMT ranged from 0.8 to 1.24 cm^3 while the mean tumor diameter, at treatment, for 13762 tumors ranged from 2.5 to 3.2 cm. The results show that 170 mg/kg of Cp was quite ineffective in the C3H/HeJ SMT. Only 14% volume regression was noted, with treatment day size re-established by Day 3. Cp at 300 mg/kg was much more effective in reducing tumor volume and resulted in approximately 50% regression with regrowth beginning on Day 6. Pretreatment size was re-established by Day 11. Tumor doubling times (T_d), calculated during the regrowth phase of the response (5 days), were shorter than those observed prior to treatment (12.4 days).

The 13762 RMT was very sensitive to Cp. Eighty mg/kg of Cp resulted in approximately 75% volume regression with regrowth evident by Day 14. The regrowth curve was similar to that for untreated, size-matched controls with pretreatment tumor size being re-established by Day 28.

Figure 2 shows the effect of 170 mg/kg of Cp on the ^{3}H-TdR LI, the PDPI, and the T_S for C3H/HeJ SMT. Aside from variations in the T_S, little or no effect on the cell kinetics was observed during the first 24 hours. At 48 hours, however, the PDPI increased to 28% while the T_S was reduced to approximately 8 hours. At 72 hours, the PDPI decreased to approximately 12%, the ^{3}H-TdR LI remained stable, and the T_S was shortened to approximately 7 hours. By 7 days, control values for all parameters were re-established.

Figure 3 shows the effect of 300 mg/kg of Cp on the PDPI and ^{3}H-TdR LI in the SMT. Within 24 hours after treatment, the PDPI and the ^{3}H-TdR LI were increased to 67.4% and 14.3%, respectively. Both parameters remained elevated for 24 hours, after which a decrease in both parameters was observed. A secondary increase in both parameters was noted on Days 4 and 5. These increases were in turn closely followed by the initiation of tumor regrowth.

Figure 4 shows the response of the PDPI and ^{3}H-TdR LI to Cp (300 mg/kg) and adriamycin (2 mg/kg) given together. The response is very simi-

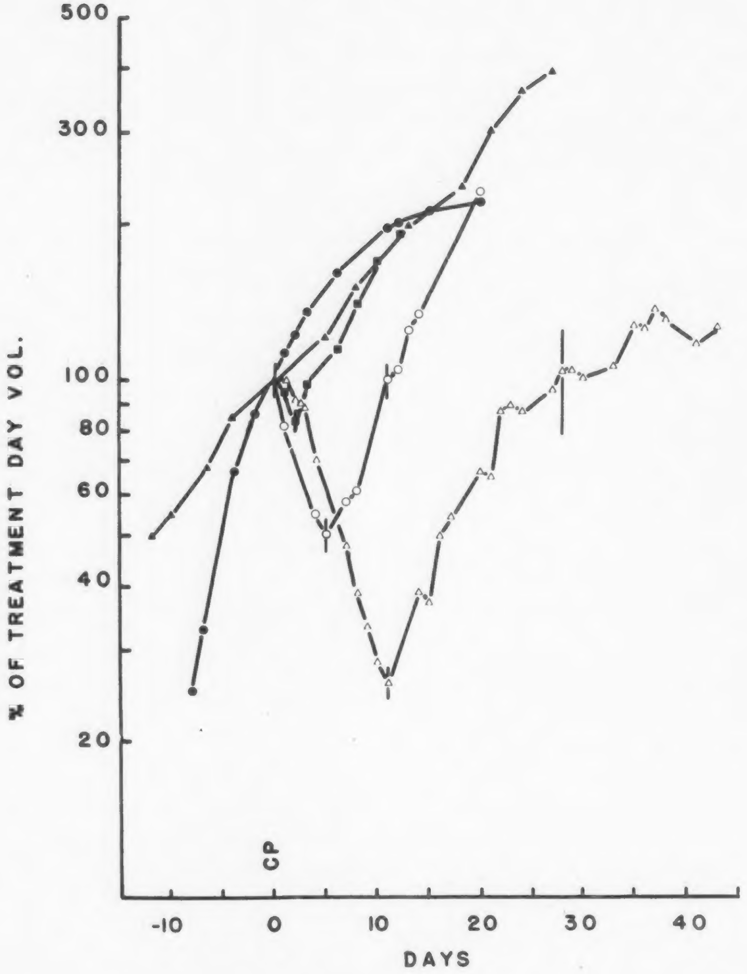

FIGURE 1.—Effect of single dose of Cp on tumor volumes for C3H/HeJ SMTs and 13762 RMTs. —▲— = untreated control growth curve for 16 SMTs; —●— = untreated control growth curve for 8 13762 RMTs where t = 0 is 14 days after transplantation; --■-- = Cp (170 mg/kg) in C3H/HeJ SMTs where each point is the mean value for 6 tumors; ---○--- = Cp (300 mg/kg) in C3H/HeJ SMTs where each point is the mean value for 8 tumors; —△— = Cp (80 mg/kg) in 13762 RMTs where each point is the mean value for 5 tumors. Tumor sizes have been normalized to the treatment day (Day 0) size.

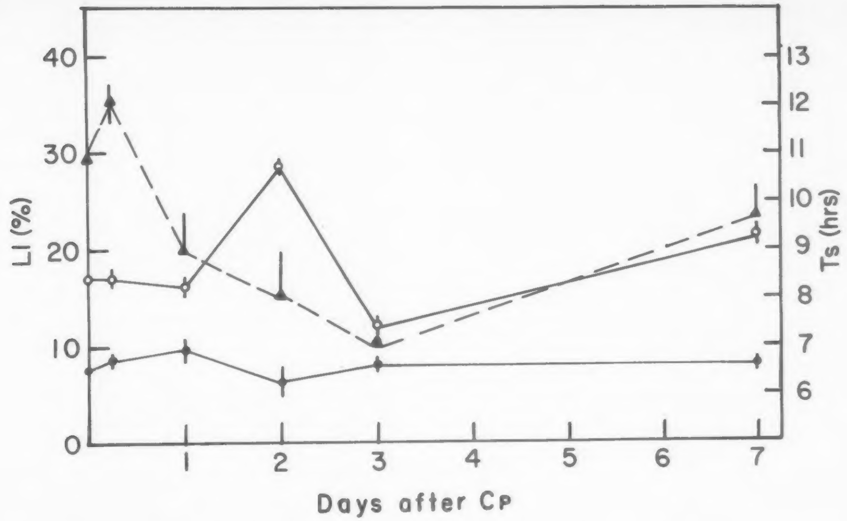

FIGURE 2.—Effect of Cp (170 mg/kg) on the PDPI (○), ^{3}H-TdR LI (●), and T$_S$ (▲) in C3H/HeJ SMT. Each point = mean ± 1 SE for 4 tumors.

lar to that seen with Cp alone; however, increases in the PDPI at 24 hours and in PDPI and ^{3}H-TdR LI at 4 days were not as profound as increases observed with Cp alone. Both parameters were, however, significantly higher than those observed for untreated controls. The reduction in tumor volume after Cp and adriamycin (44% ± 5%) was not different from that with Cp alone (51% ± 6%).

To determine if the increased ^{3}H-TdR LI and PDPI at 24 hours after Cp (300 mg/kg) could indicate increased sensitivity of the cell population to adriamycin, the tumor growth and cell kinetic perturbations after Cp and adriamycin (24 hours after Cp) were determined and are shown in figure 5. Within 24 hours after adriamycin, the ^{3}H-TdR LI was decreased. Subnormal ^{3}H-TdR LIs were observed until Day 4 when the ^{3}H-TdR LI was increased to approximately 17%. Unlike Cp alone, however, the PDPI continued to increase,

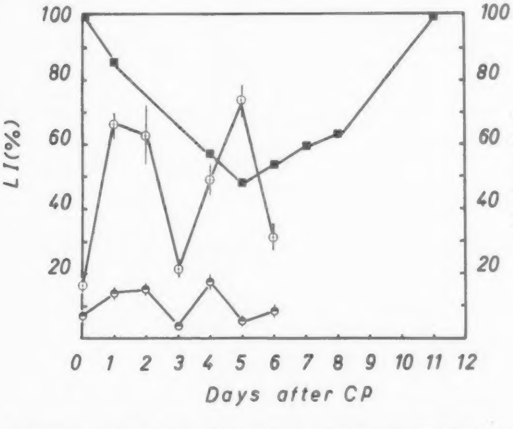

FIGURE 3.—Effect of Cp (300 mg/kg) on PDPI (⊕), ^{3}H-TdR LI (◓), and tumor volume (Vt) normalized to the treatment day volume (V0) and expressed as a % (■) in C3H/HeJ SMT. Each point = mean ± 1 SE for 4 tumors.

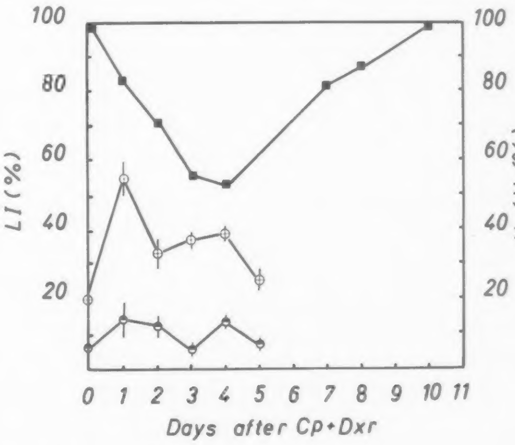

FIGURE 4.—Effect of Cp (300 mg/kg) and adriamycin (2 mg/kg) given together on the PDPI (⊕), ^{3}H-TdR LI (◓), and tumor volume (Vt) normalized to the treatment day volume (V0) and expressed as a % (■) in C3H/HeJ SMT. Each point = mean ± 1 SE for 4 tumors.

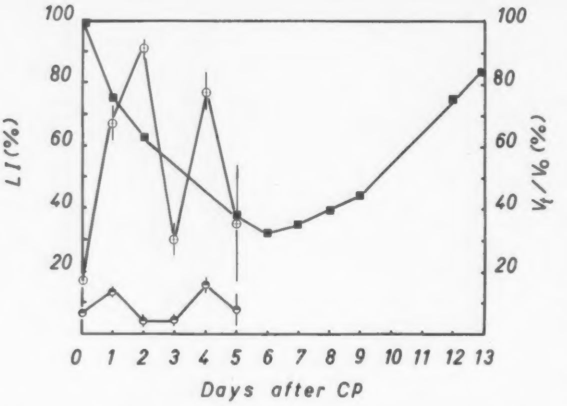

FIGURE 5.—Effect of adriamycin (2.0 mg/kg) given 24 hrs after Cp (300 mg/kg) on the PDPI (⊕), ^{3}H-TdR LI (●), and tumor volume (Vt) normalized to the treatment day volume (V0) and expressed as a % (■) in C3H/HeJ SMT. Each point = mean ± 1 SE for 4 animals.

until by Day 2 approximately 90% of the cells were PDP positive. Increases in PDPI and ^{3}H-TdR LI, presumably indicating proliferative recovery, were noted 2–3 days prior to the initiation of tumor regrowth. The addition of adriamycin at 24 hours, when both the ^{3}H-TdR LI and PDPI were increased, resulted in approximately 73% ± 6% tumor volume regression as compared to approximately 51% ± 6% and 44% ± 5% for Cp alone and for simultaneous Cp plus adriamycin, respectively.

Unlike the C3H/HeJ mammary tumor system, the 13762 RMT is a high growth fraction, rapidly proliferating tumor model. Figure 6 shows the effect of 80 mg/kg of Cp on the cell kinetics of this tumor system. Within 6 hours after Cp, the ^{3}H-TdR LI was depressed to 10.3% ± 0.4%. By 24 hours after Cp, the PDPI was slightly increased. Although still subnormal, the ^{3}H-TdR LI at 24 hours was significantly higher than the 6-hour values. During the next 6 days both the PDPI and ^{3}H-TdR LI decreased until, by Day 7, mean values for PDPI and ^{3}H-TdR LI were 26.7% and 2.4%, respectively. The T_Ss were prolonged to approximately 10 hours between Days 2 and 7. Between Days 8 and 11, PDPI, ^{3}H-TdR LI, and T_Ss returned to control levels. On Day 11, mean values for PDPI, ^{3}H-TdR LI, and T_S suggested a more rapid cell proliferation than in untreated, age-matched controls in which mean values for mean and standard error values for the PDPI, the ^{3}H-TdR LI, and the T_S were 64.5% ± 3.5%, 27.6% ± 1.8%, and 5.6 ± 0.2 hours, respectively. Proliferation on Day 11 was, however, similar to cell proliferation prior to treatment. Recovery of the kinetic param-

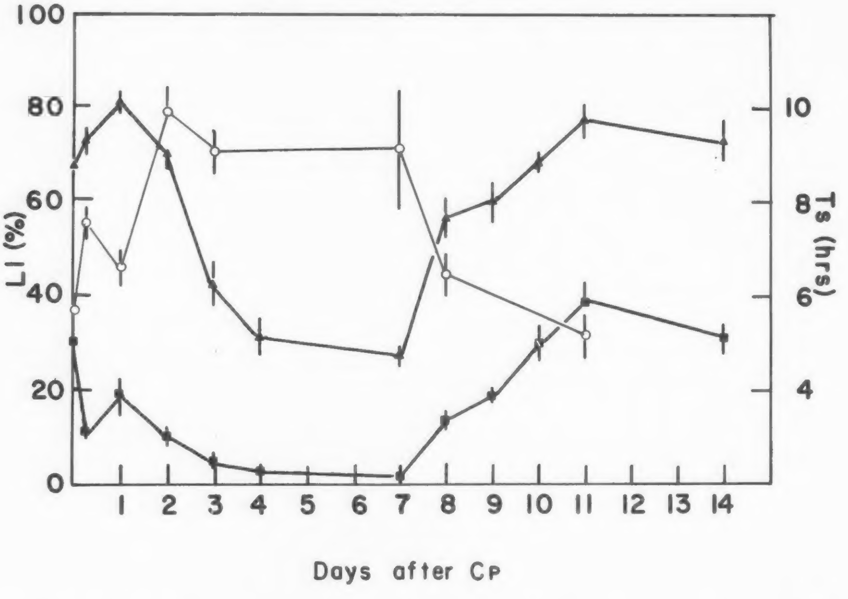

FIGURE 6.—Effect of 80 mg/kg of Cp on the PDPI (▲), ^{3}H-TdR LI (■), and T_S (○) in 13762 RMT. Each point = mean ± 1 SE for 4 tumors.

eters was observed within 2–3 days prior to initiation of tumor regrowth observed on Day 14 (*see* fig 1).

These results would suggest that for sequencing subsequent cycle-active agents in this tumor, two intervals may be advantageous: Day 1 when the ^{3}H-TdR LI shows a transient increase and between Days 9 and 11 when both the PDPI and ^{3}H-TdR LI are recovering. Table 2 shows some results from studies in which Cp and adriamycin were used sequentially for treatment of the 13762 RMT.

In these studies, treatment was initiated on Day 14 after transplantation. Animals were adjudged to be in complete remission (CR) if tumors became nonpalpable and were subsequently designated as long-term survivors (LTS) if they remained tumor-free for at least 100 days. Mean survival was calculated for animals not reaching LTS status.

Single-dose Cp (80 mg/kg) resulted in 25% remission, only 5% LTSs, and only a marginal increase in overall survival (protocol 2). Two Cp treatments given on Days 14 and 23 (protocol 3) resulted in 80% remissions, 30% LTSs, and significantly increased survival for non-LTS animals. While three consecutive daily Cp treatments (protocol 5) to a similar total Cp dose resulted in a similar remission frequency and more LTSs, the additional toxicity attendant with this schedule was reflected by the mean survival of non-LTS animals. When the second Cp dose was fractionated between Days 22 and 24 (protocol 6), twice as many LTSs as in protocol 3 were noted with a similar total Cp dose. The results from these studies with 150–170 mg/kg of Cp can be compared to the results with protocol 4 where 160 mg/kg of single-dose Cp was relatively ineffective. Protocols 7 and 8 show results from studies where 240 mg/kg of Cp is given either as a single dose or fractionated as 80 mg/kg, every 9 days × 3. The results indicate that, in this tumor system, the fractionated Cp schedules designed on the basis of the observed cell kinetic perturbations were more effective than single-dose treatments.

Cp and adriamycin were used in combination as described by protocols 9–17. In studies with single Cp and adriamycin treatments, remission frequencies and survival were similar to those observed after Cp alone (protocol 2). Simultaneous Cp and adriamycin had little or no increased benefit.

Combination drug studies with fractionated Cp and adriamycin are described in protocols 13–17. In these studies, Cp given every 9 days × 2 was used as a foundation for combination therapy. The addition of adriamycin, given simultaneously with Cp (protocol 17), was, in terms of LTS, less effective than Cp alone (protocol 3). When adriamycin was given 1 day after Cp, to exploit the transient increases in both LI and PDPI, CRs and LTSs were noted in all animals (protocol 15). In contrast, when adriamycin was given 6 hours after each Cp dose, a time when the ^{3}H-TdR LI was shown to be low, remission frequency and LTSs were similar to Cp alone.

DISCUSSION

The present studies were initiated to evaluate the cell kinetic perturbations induced by Cp in C3H/HeJ SMT and 13762 RMT. Within the first 24 hours after Cp (300 mg/kg), increases in both ^{3}H-TdR LI and PDPI relative to control were noted. In the 13762 model, although ^{3}H-TdR LI was subnormal at 24 hours after Cp (80 mg/kg), a transient increase relative to the 6-hour value was noted. In both tumor models, intervals of decreased proliferation were noted. In the 13762 model, recovery of the ^{3}H-TdR LI and PDPI was noted prior to the initiation of regrowth. Although the data are not as conclusive for C3H/HeJ SMT, a similar relationship between recovery of the PDPI and ^{3}H-TdR LI may exist.

Although the PDPI has been shown to estimate the growth fraction in unperturbed, experimental tumors (22,25), the precise relationship of this measurement to the growth fraction in the perturbed state is not known. Both the PDPI and ^{3}H-TdR LI were increased within the first 24 hours after 300 mg/kg of Cp in the C3H/HeJ SMT. The increased PDPI may be reflective of rapid recruitment of noncycling tumor cells. The prompt tumor volume regression after Cp and the delayed tumor regrowth could, however, suggest that the increased ^{3}H-TdR LI and PDPI observed during the first 38 hours after Cp were reflective of cells capable of only limited division. But, since small doses of adriamycin, given 24 hours after Cp, resulted in enhanced tumor regression, more regrowth delay, and suppression of tumor cell proliferation (decreased ^{3}H-TdR LI), the early increases in ^{3}H-TdR LI and PDPI were probably not totally reflective of lethally damaged cells.

Although the cell kinetic perturbations in the high growth fraction 13762 tumor were qualitatively similar to those in the low growth fraction C3H/HeJ tumor model, important differences were noted. For example, although the PDPI was slightly increased at 24 hours, the ^{3}H-TdR LI was substantially subnormal. Further, and perhaps more important for treatment planning, the interval during which proliferation was suppressed (ie, the period of subnormal PDPI and ^{3}H-TdR LI) was considerably longer in the 13762 tumor than in the C3H/HeJ tumor. In

TABLE 2.—Treatment efficacy of protocols designed from the cell kinetic perturbations after Cp in 13762 rat tumors

Protocol No.		Cp			Adriamycin			CR (%)	LTS† (%)	MS‡ (days)
		Days*	mg/kg/dose	Total dose	Days*	mg/kg/dose	Total dose			
1	Control	—	—	—	—	—	—	0	0	32.6
2	Cp	14	80	80	—	—	—	25	5	50.6
3	Cp	14, 23	80	160	—	—	—	80	30	88.9
4	Cp	14	160	160	—	—	—	0	0	73.0
5	Cp	14, 15, 16	50	150	—	—	—	78	44	54.3
6	Cp	14	80							
	Cp	22–24	30	170	—	—	—	100	66.7	90.4
7	Cp	14, 23, 32	80	240	—	—	—	100	100	—
8	Cp	14	240	240	—	—	—	25	25	85.3
9	Cp + adriamycin	14	80	80	14	2.0	2.0	25	0	65.3
10	Cp + adriamycin	14	80	80	16	1.0	1.0	50	25	46.0
11	Cp + adriamycin	14	80	80	17	1.0	1.0	25	0	43.7
12	Cp + adriamycin	14	80	80	18	1.0	1.0	25	0	55.2
13	Cp + adriamycin	14, 23	80	160	32–35	0.5	2.0	100	75	56.0
14	Cp + adriamycin	14, 23	80	160	24, 25	1.0	2.0	100	100	—
15	Cp + adriamycin	14, 23	80	160	15, 24	1.0	2.0	100	100	—
16	Cp + adriamycin	14, 23	80	160	14.25, 23.25§	1.0	2.0	75	25	79.3
17	Cp + adriamycin	14, 23	80	160	14, 23	1.0	2.0	100	0	81.2

* Days after transplantation.

†>100-day survivors.

‡Mean survival in days of animals not achieving LTS.

§Adriamycin given 6 hrs after Cp on Days 14 and 23.

the 13762 tumor, maximal PDPI and ^{3}H-TdR LI reductions were seen 7 days after Cp. The data are compatible with a T_C of approximately 70 hours if exponential growth is assumed (29) or 108 hours if the cell population is in a steady state.

Another important difference between the two models was the observation that the PDPI in 13762 tumors did not increase significantly above pretreatment control values, which could imply little or no additional recruitment of noncycling cells. This idea is supported by the observation that the posttreatment regrowth curve was similar to the pretreatment regrowth curve. In contrast, the increases in PDPI and ^{3}H-TdR LI observed prior to regrowth in the C3H/HeJ SMT, together with the more rapid growth rates in regrowing tumors, are more indicative of recruitment of noncycling tumor cells. Increases in both the ^{3}H-TdR LI and growth fraction have been previously noted during the rapid regrowth phase following radiation. Subsequent treatment at these times resulted in greater tumor volume regression than did a similar treatment in non-irradiated tumors (30).

Chemotherapy schedules for the 13762 tumor with Cp and Cp plus adriamycin to exploit the cell kinetic perturbations, were designed and tested. Although some LTSs could be obtained with single-dose Cp, schedules with sequential Cp administration every 9 days, an interval corresponding to the time required for PDPI and ^{3}H-TdR LI recovery, were superior to schedules where an equivalent total Cp dose was given singly. The addition of a small dose of adriamycin 24 hours after Cp, when the ^{3}H-TdR LI was significantly increased from the 6-hour value, resulted in LTSs after only two courses. In contrast, two courses of Cp every 9 days with adriamycin given either together with or 6 hours after Cp were no more effective than Cp alone. Interestingly, Cp and adriamycin given simultaneously every 9 days, while being as effective as Cp alone in producing CRs, were less effective in producing LTSs. The addition of adriamycin may, however, have altered the interval for LI and PDPI recovery enough to make the 9-day interval inappropriate. Adriamycin, given 24 hours after Cp, was also shown to be synergistic in L1210 cells (31).

It is difficult to reconcile the survival results in 13762 tumors given adriamycin at 6 and 24 hours after Cp in terms of the observed twofold difference in ^{3}H-TdR LI, especially in light of the results in the C3H/HeJ SMT where adriamycin given at a time when the ^{3}H-TdR LI was increased twofold relative to controls resulted in only a 40% increase in tumor regression. A comparison such as this, however, may not be valid due to the differences in drug dose, drug sensitivity, and unperturbed cell kinetics in these two models. Within the context of each tumor model, changes in ^{3}H-TdR LI, while not necessarily predicting the magnitude of response, did seem to indicate an interval of increased adriamycin sensitivity. C3H/HeJ SMT showed elevated ^{3}H-TdR LIs and increased PDPIs at 24 and 48 hours after 300 mg/kg of Cp, which together could indicate an increase in the size of the cycling cell population. The relatively transient change in ^{3}H-TdR LI in the high growth fraction 13762 tumor after 80 mg/kg could, on the other hand, indicate an interval of partial cell synchronization. While the proximity of the study intervals is not close enough to discern synchronization, differential effects such as these in the two tumor models might explain why adriamycin at 24 hours after Cp could be more effective in the 13762 model than in the C3H/HeJ SMT. Other factors, including differences in drug distribution, repair capacity, and cell clearance in the two model systems, may, together with the cell kinetics, also be important determinants of response.

The results with Cp in the 13762 tumor are reminiscent of the response to BCNU in L1210 leukemia (32). In those studies, ^{3}H-TdR LIs were decreased within 1 day after treatment and remained depressed for approximately 8.5 days. Although T_Ss were not significantly prolonged after Cp in L1210, BCNU treatment did result in a doubling of the T_S. In a more recent study with BICR A15 carcinomata in Augest rats, Cp (100 mg/kg) resulted in subnormal ^{3}H-TdR incorporation for at least 10 days after treatment (33). Previous studies in our own laboratory with T1699 mammary tumors have shown that the PDPI could predict when, following a single dose of vincristine, these tumors were maximally sensitive to Cp (34). In these studies, longer survival and more cures were observed when Cp was administered after vincristine when the PDPI was increased relative to untreated controls. The results from the present studies are also consistent with the paradigm suggested by Schenken (9) which proposes that efficacious sequential chemotherapy can be based on the proliferative response of the tumor to the perturbing effects of chemotherapeutic agents.

The control mechanisms regulating experimental tumor proliferation following treatment are not known and are presumably multifaceted. Although the changes in cell kinetics after treatment can be a direct result of the action and biologic half-life of the drug used, resumption of cell proliferation, as indicated by changes in the cell kinetics, may also reflect host-mediated events including, but not limited to, the production of humoral stimulatory factors (35). The changes in cell kinetics following Cp treatment were shown to be useful in predicting when, following treatment, the tumor cell population might be sensitive to subsequent treatments. In the 13762 tumor system, these cell kinetic changes were a useful foundation upon which effective sequential and combination chemotherapy schedules could be designed. Other studies are currently underway with other drugs in these tumor systems to further evaluate our in vitro methods as prognostic indicators of tumor chemosensitivity.

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Effect of a Transplantable Leukemia on Hematopoietic Stem Cells in AKR Mice[1,2]

Alexander Nakeff* and Frederick A. Valeriote[3]

SUMMARY

The number of colony-forming units-spleen (CFU-S) and colony-forming units-culture in the femoral marrow of mice bearing an AKR transplantable leukemia remained relatively unchanged up to the death of the host despite the almost complete displacement of the normal differentiated hematopoietic cells by infiltrating leukemia cells. The proliferative state of the CFU-S was not changed significantly by the presence of the leukemia cells as determined by using 5-fluorouracil as a proliferative probe. The implications of these findings are discussed with regard to possible control mechanisms governing the interaction of leukemia cells with normal marrow cells and their potential importance for cancer chemotherapy.

[Cancer Treat Rep 62:737–741, 1978]

Knowledge of the interaction between leukemia cells and normal hematopoietic progenitor cell populations is of fundamental importance to our understanding of normal regulatory mechanisms and the manner in which leukemia affects hematopoiesis. In addition, knowledge of the cellular basis of this interaction is important to the rational use of various chemotherapeutic agents since it is necessary that normal progenitors survive the chemotherapy and eventually repopulate hematopoietic tissue to restore hematopoiesis.

It is generally believed that, as a leukemia cell population invades and proliferates in the bone marrow, it crowds out or replaces the normal hematopoietic progenitor cells (1). Although the progressive loss of normal differentiated hematopoietic elements as a function of the extent of invasion of marrow by leukemia cells has generally been reported in various experimental leukemia systems (2–6), the effect of proliferating leukemia cells on the number of myeloid-committed progenitor cells or colony-forming units-culture (CFU-C) has been contradictory (2–6). With regard to the number of pluripotent stem cells assayed as colony-forming units-spleen (CFU-S) in leukemic marrow, the results are also contradictory in the L1210 leukemia model (7) and the AKR spontaneous leukemia model (8). In vivo assays were not done as a function of the course of the leukemia since both the leukemia cells and the CFU-S give rise to macroscopic colonies, thereby rendering the assay uninterpretable at late times.

This report presents data on the effect of a transplanted leukemia on CFU-S and CFU-C in the bone marrow of mice, showing that the number of these normal hematopoietic progenitors did not change significantly throughout the course of the leukemia. In addition, data are presented using 5-fluorouracil (5-FU) as a proliferative probe (9), indicating that the CFU-S present in the leukemic marrow remain in a "resting" or nonproliferating state.

MATERIALS AND METHODS

Mice.—Female AKR mice (National Laboratories, St. Louis, Mo, or Jackson Laboratories, Bar Harbor, Me), 9–14 weeks old, were used throughout these studies.

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Leukemia cells.—A transplantable AKR leukemia (10) initially derived as a lymphoma (11) was used for this study, as obtained originally from Dr. Robert Bruce at the Ontario Cancer Institute (Toronto, Canada). In our laboratory, the leukemia has been passed weekly for > 7 years in AKR mice and is lethal to recipients 5–6 days after the iv injection of 10^6 leukemia cells.

CFU-S assay.—Mice were injected iv with 10^6 cells obtained from the spleens of AKR leukemia donors. Daily thereafter, groups of three recipient mice were injected with a single dose of 2 mg of cyclophosphamide (CY) which has been shown to be sufficient to decrease the surviving fraction of leukemia cells to 10^{-7} but to have little effect on CFU-S (12). Two hours after the administration of CY, the mice were killed by cervical dislocation and a monodispersed cell suspension was prepared from their pooled femoral marrow in phosphate buffered saline (PBS) as previously described (13). An appropriate fraction of the suspension was injected via the tail vein into groups of ten syngeneic recipients that had received a total dose of 1000 rads of ^{137}cesium gamma rays. The dose was delivered in two 500-rad exposures administered 3 hours apart as described by Hellman and Grate (14). Nine days later, the mice were killed, their spleens were harvested and placed in Bouin's solution, and the macroscopic spleen colonies were counted. The number of CFU-S was expressed per femur without the consideration of an "f" factor. Leukemic spleen colonies, which are much larger than normal spleen colonies, were not observed in these studies.

CFU-C assay.—At daily intervals after the iv injection of 10^6 leukemia cells, groups of three recipient mice were killed and their bone marrow was collected as described above. Viable nucleated cells were counted electronically in cetrimide following a 10-minute digestion in pronase to lyse nonviable cells (15). Nine cultures were made using a modified thin-layer technique as described by Dicke et al (16). A bottom layer of 2.5 ml of a 0.5% (vol/vol) solution of agarose (Seakem, Microbiological Associates) in alpha-MEM (17) with 10% horse serum (Gibco) and 10% L-cell conditioned medium was allowed to gel in each of four quadrants of a Falcon No. 1001 disposable petri dish. Over this, an upper layer of 0.25 ml of a 0.25% (vol/vol) agarose solution in alpha-MEM containing either 1 or 2×10^4 viable nucleated cells with 10% horse serum was layered and allowed to gel at room temperature before being cultured for 7 days at 37°C in a humidified atmosphere at 7.5% CO_2 in air. CFU-C were counted using an inverted microscope with magnification × 40 and the number was expressed per femur. The leukemia cells never formed colonies in this assay system following the plating of up to a tenfold larger number of cells from enlarged, leukemia-bearing spleens.

Bone marrow cell morphology.—A 50-μl aliquot of bone marrow was sedimented quantitatively onto glass slides using a Sayk apparatus (18) and stained with May-Grünwald-Giemsa. A minimum of 100 cells were counted with magnification × 400 over several random areas and the number of leukemia (blast) cells expressed as a percent of the total cells was counted.

Proliferative state of CFU-S.—Mice were injected with 10^6 leukemia cells and at various times thereafter they, as well as normal mice, were given 5 mg of 5-FU (Hoffmann-LaRoche, Nutley, NJ) ip. The marrow was harvested 4 hours after each 5-FU administration and the equivalent of one-half femur was injected into groups of 15 lethally irradiated recipients and assayed for CFU-S as described previously. This dose of 5-FU was chosen because it has been shown to decrease normal CFU-S survival to about 10^{-2} while reducing the leukemia cell population to undetectable levels (9,12). Further, this agent is very effective upon proliferating CFU-S and a dose of 5 mg would be expected to reduce the surviving fraction to undetectable levels as well (9). Thus, 5-FU can be used as a probe to determine the proliferative state of CFU-S. The greater the fraction of proliferating CFU-S, the lower the survival would be. No leukemia cell colonies were observed in these spleens at the dose of 5-FU used.

RESULTS

The percentage of leukemia cells and the number of CFU-S and CFU-C per femur as a function of time after the transplantation of 10^6 leukemia cells are presented in figure 1.

As shown in figure 1A, the proportion of leukemia cells in the bone marrow did not change appreciably over the first 3 days after transplantation. Thereafter, the number of normal, differentiated bone marrow cells continued to decrease over that present in control (nonleukemic) bone marrow while blast-like leukemia cells became more and more frequent. Since the total cellularity per femur did not change significantly throughout the entire course of the leukemia, it would appear that the proliferating leukemia cells "pack" the marrow cavity and displace the normal differentiated cells. Although the leukemic lymphoblast cells, being large and undifferentiated, were relatively easy to identify morphologically, there was some difficulty in identifying them when their numbers were relatively low. A measure of this is reflected in the average of 10% "leukemia-like" cells present in

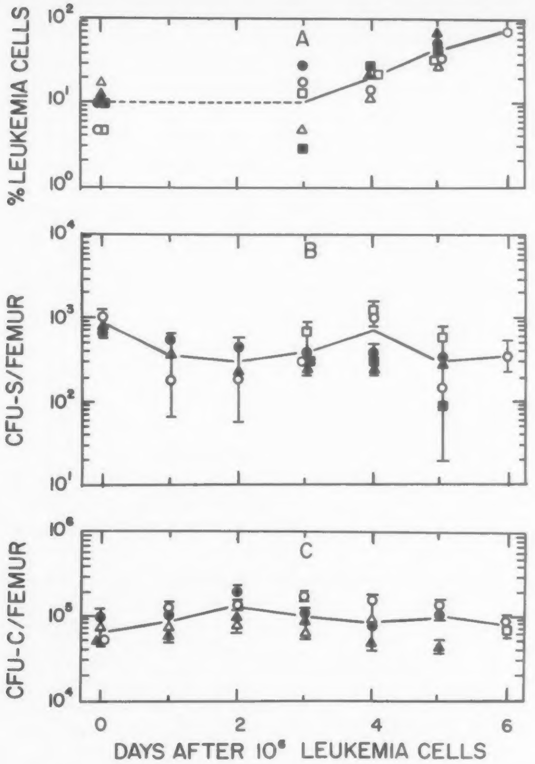

FIGURE 1.—No. of leukemia cells (A), CFU-S (B), and CFU-C (C) in the femurs of AKR mice as a function of time following the iv administration of 10^6 leukemia cells. Each symbol represents a separate experiment and errors shown represent ± 1 SE. The number of CFU-S at Time 0 was obtained from control, non-leukemia-bearing mice 2 hrs after the administration of 2 mg of CY.

normal bone marrow (at Day 0). We used a morphologic criterion derived from the examination of large numbers of leukemia cells obtained from the spleens of leukemia-bearing donors just prior to their death, at a time when almost all the spleen cells are known to be leukemia cells. After Day 3, the number of leukemia cells in the bone marrow increased almost exponentially until, by Day 6 in one experiment, the leukemia cells constituted almost 90% of the bone marrow cellularity.

Within 2 hours after the administration of CY to control (nonleukemic) mice, about 20% of the CFU-S were killed with a decrease to 0.90 (± 0.05) × 10^3/femur from 1.14 (± 0.1) × 10^3/femur in normal untreated mice (fig 1B). This result is in agreement with previous drug studies (12). Twenty-four hours after leukemia cell transplantation, however, this dose of CY reduced the surviving fraction of CFU-S to approximately 25%, that is, 75% of the CFU-S were killed by the CY (fig 1B). Throughout the remaining course of the leukemia, the surviving fraction of CFU-S obtained following the CY treatment remained between 25% and 80%.

TABLE 1.—5-FU sensitivity of CFU-S in femoral marrow of leukemic mice

	Surviving fraction	
Group	Experiment 1	Experiment 2
Control*	1.0 (9.6 × 10^2)†	1.0 (9.3 × 10^2)
2-hr	4.3 × 10^{-2}	8.7 × 10^{-2}
1-day	1.1 × 10^{-1}	3.3 × 10^{-2}
4-day	8.7 × 10^{-2}	
5-day	6.8 × 10^{-2}	

*This group received neither the leukemia cells nor the 5-FU.
†Nos. in parentheses = absolute No. of CFU-S.

The average number of CFU-C (fig 1C) remained relatively constant at a level that was either at or above normal throughout the entire course of the leukemia. The normal level is found by us to be 1.64 (± 0.20) × 10^4 in AKR mice.

Finally, the proliferative state of the CFU-S in the leukemia-bearing mice was assessed by examining the surviving fraction of CFU-S following a single dose of 5-FU administered to normal mice and to mice that had received 10^6 leukemia cells. As shown in table 1, the surviving fraction of the CFU-S, compared to an untreated control, remained unchanged as a function of the time the host carried the leukemia, ie, as a function of the degree of packing of the marrow with leukemia cells. The degree of CFU-S killing in the leukemia-bearing hosts was the same as that reported in normal AKR mice given the same dose of 5-FU (12).

DISCUSSION

Using a rapidly proliferating, transplantable leukemia in AKR mice, we observed no significant change in the number of CFU-S and CFU-C in the marrow of leukemia-bearing hosts despite the obvious "packing" of the marrow with leukemia cells and the resultant displacement of normal, differentiated hematopoietic cells.

The "crowding-out" of normal differentiated hematopoietic cells in marrow by proliferating leukemia cells seems to be a general phenomenon, as reported in other leukemia cell systems (4,6), although conflicting results have been reported for quantitative changes in CFU-C which seem to reflect the type of leukemia being studied. Chevalier et al (3) have shown a decrease in the number of CFU-C present in the marrow of AKR mice with spontaneous lymphoma, whereas Hibberd and Metcalf (2), using the same system, reported little correlation between the number of CFU-C and the degree of invasion of the marrow with lymphoma. The latter investigators did demonstrate increases in the CFU-C content of marrow (and much more for spleen) in mice bearing a variety of transplantable tumors. Khaitov et al (19) showed that CFU-C increased substantially in bone marrow during the early phases of tumor growth but were markedly depressed as the tumor progressed for a number of different tumors. However, the number of endogenous spleen CFU-S increased with tumor growth. Finally, using a virus-accelerated AKR leukemia, an increase in CFU-C in marrow has been shown with progression of the disease (20). These same authors also reported that a passaged leukemia (10^5 cells killing in about 14 days) caused a significant increase in CFU-C/10^4 nonleukemia cells. Using a transplantable myeloid leukemia in RFM mice having a similar time course of lethality as the transplantable lymphoma used in our studies, Gordon and Coggle (5) have presented results similar to ours for CFU-C. In rats, Handler et al (4) and Hoelzer et al (6) have used a transplantable myelogenous (Shay) leukemia and a myelomonocytic leukemia, respectively, both of which are chemically induced; they showed an increase and a decrease, respectively, in the number of CFU-C in bone marrow, which correlated with the progression of the disease. In the latter study, the failure of normal hematopoiesis in rats with a rapidly growing leukemia was demonstrated by the rapid onset of anemia and thrombocytopenia in the terminal state (6 days after transplantation) when 50% of the marrow has been replaced by leukemia cells.

Handler and Handler (21) extended this study to show a large reduction (about tenfold) of CFU-E (colony-forming units-erythroid) in rat bone marrow with progression of the Shay chloroleukemia that was not due simply to dilution of the cells. However, there seemed to be a compensatory increase in spleen CFU-E during the course of the leukemia.

In the studies of human marrow, while CFU-C decreased in number with progression of the disease and returned to normal levels during remission (22), the effect on more primitive progenitors is presently unknown.

In the only complete study (6) devoted to assaying changes in pluripotent cell progenitors as a function of leukemia cell infiltration, a complex tritiated-thymidine labeling technique was used to follow "labeled stem cells" which were assumed to be pluripotent. The number of these "stem" cells showed only a modest decrease in bone marrow as a function of the degree of invasive myelomonocytic leukemia present in the marrow. These results are similar to those we obtained using the spleen colony assay.

In order to interpret the finding that the number of CFU-S and CFU-C showed no significant change with the progression of leukemia in the AKR mouse model, it is important to know the proliferative state of these progenitor populations. In light of the fact that the normal differentiated cells clearly decreased within a few days after transplantation of the leukemia, differentiation from the progenitor compartments may have ceased, in which case most, if not all, of the CFU-S and CFU-C would be expected to be out of cell cycle. Alternatively, CFU-S and CFU-C may have been continuously lost from their respective compartments due to displacement by leukemia cells, with that loss being compensated for by their increased recruitment into cell cycle in a sufficient magnitude to maintain the normal degree of cellularity within their respective cell compartments. The data using 5-FU as a cycle-specific probe to test directly for the proliferative state of the CFU-S tend to support the former model, since it is clear at least that the CFU-S present in leukemic marrow are not proliferating at a rate greater than that found in normal marrow. If these CFU-S were proliferating, we would have expected them to become much more sensitive to cell killing by 5-FU, with an undetectably low surviving fraction at the dose of 5-FU employed here (9). The slightly lower number of surviving CFU-S from leukemic marrow as compared to normal marrow may have simply reflected the lower number of CFU-S in the leukemic marrow observed prior to 5-FU administration. Additional support for the lack of proliferative activity in normal progenitors comes from the study of Hoelzer et al (6) in which the constancy in the labeling index of their "labeled stem cells" present in a packed leukemic marrow was interpreted as indicating the lack of proliferative activity in a class of "candidate" pluripotent stem cells.

A possible model for the effect we observed is that the proliferating leukemia cells act to contact-inhibit the CFU-S and CFU-C and effectively block the input of new cells into the differentiated compartments. At the same time, those cells further down the pathway of differentiation than the committed progenitor cells mature normally and leave the marrow, thus producing the decrease in normal bone marrow cells and cell functions observed by us and others (6,23). There is clinical evidence for such a model in human leukemia (24).

The implication of the results with regard to chemotherapy is potentially quite significant. If a tumor-cell-packed marrow does not imply a significant decrease in the normal hematopoietic stem cell population, full courses of therapy might not then be counter-indicated. Further, if the stem cells are out of cycle as well, they might be even less sensitive to the cytotoxic effects of the drugs than the normal marrow.

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Therapeutic Value of Mepitiostane in the Treatment of Advanced Breast Cancer [1,2]

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SUMMARY

A new, orally active antiestrogenic steroid, mepitiostane (20 mg/day), was given to 45 patients with advanced breast cancer. The regression rate was 31.1%, or 14 of 45 patients, and a duration of regression of > 6 months was obtained in seven patients. Virilizing effects such as hoarseness, hirsutism, and acne were observed relatively often, but there was no evidence of abnormality in the liver function tests or in the serum calcium level.

[Cancer Treat Rep 62:743–745, 1978]

It has been reported that a new steroidal antiestrogen, epitiostanol ($2\alpha,3\alpha$-epithio-5α-androstan-17β-ol), given im to patients with advanced breast cancer produced a better regression rate and fewer virilizing side effects than testosterone propionate (1). Mepitiostane, a derivative of epitiostanol, when given orally has been known to possess potent antiestrogenic and antitumor (mammary tumor) activities similar to those of epitiostanol with much less hepatotoxicity as compared with 17α-alkylated steroids (2–4).

A preliminary clinical trial of mepitiostane using various doses in 41 patients with advanced breast cancer showed a 26.8% (11 of 41 patients) objective remission rate, with the average duration of remission being 10.5 months (5). The present study was designed to evaluate the therapeutic benefits of this new oral steroidal antiestrogen in patients with advanced breast cancer.

MATERIALS AND METHODS

The structure of mepitiostane is shown in figure 1. The drug was administered orally at a dose of 20 mg/day to patients with advanced breast cancer. From January 1975 to December 1976, of patients with advanced breast cancer at the three institutions (those eligible according to the admission criteria of the protocol used by the US Cooperative Breast Cancer Group [6] except for menstrual status), a total of 45 were selected for this study. The patients were both pre- and post-menopausal with objective evidence of disease progression and had not received previous hormonal anticancer therapy. None of the patients had received additive hormonal therapy. Seven patients had been subjected to oophorectomy prophylactically or therapeutically, and one patient had received a minor chemotherapeutic agent 4 weeks before entering the study.

The methods of assessment were essentially identical to those of the protocol used by the Cooperative

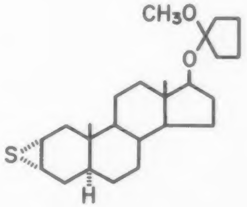

FIGURE 1.—Mepitiostane structure.

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[2] Mepitiostane: CAS reg. No. 21362-69-6; $2\alpha,3\alpha$-epithio-5α-androstan-17β-yl, 1-methoxy-cyclopentyl ether.

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TABLE 1.—Distribution of objective regression to hormonal treatment with mepitiostane

Dominant lesion	No. of patients with objective regression/No. treated Menstrual status: Premenopausal	Menopausal for— < 5 yrs	Menopausal for— > 5 yrs	Total	%
Soft tissue	0/2	3/9	2/6	5/17	29.4
Osseous	2/5	2/2	2/5	6/12	50.0
Visceral	0/4	0/1	3/11	3/16	18.8
Totals	2/11	5/12	7/22	14/45	31.1

Breast Cancer Group (6). All patients in this study were assessed by responsible members of the three institutes. During the period of study, patients were examined at intervals of 4 weeks, at which time roentgenograms of the chest, skull, dorsolumber spine, and pelvis were taken and all palpable masses were examined. In the early stage of therapy, patients were clinically examined every week. Photographs of the involved areas were also taken. The drug was administered until there was objective evidence of progression of the disease. Laboratory tests before and during therapy included routine blood cell counts, SGOT, SGPT, and serum alkaline phosphatase, protein, calcium, and phosphorus.

RESULTS

Objective regression according to site of dominant lesion and menstrual status is shown in table 1. Objective regression was obtained in 14 of 45 patients (31.1%). The duration of regression was from 1 to 16 months, with an average of 7 months. Presently, five of the patients are still in regression.

The incidence of side effects is listed in table 2. Some virilizing side effects such as hoarseness, hirsutism, and acne were relatively common. These side effects were not severe and disappeared within 3 months after cessation of drug administration. Three of the patients showed elevated levels of alkaline phosphatase during therapy, clearly due to the progression of disease. In another three patients, abnormal alkaline phosphatase levels dropped to the normal range concomitant with the regression. In the other patients, no significant change of the alkaline phosphatase levels was observed during the treatment. There were no significant changes in blood cell counts or serum protein, transaminases, calcium, or inorganic phosphorus during the treatment.

TABLE 2.—Incidence of side effects

Side effect	No. of patients
Hoarseness	28
Hirsutism	21
Acne	19
Edema	9
Nausea	2
Stiff hand	2
Pigmentation	1
Facial flushing	1
Palpitation	1
Depilation	1

DISCUSSION

Recently, antiestrogens have become an accepted addition to the traditional hormonal treatment of breast cancer. The antiestrogens most often used in breast cancer at present are tamoxifen and nafoxidin. These compounds have antiestrogenic as well as slight estrogenic activity (7), and their mechanism of action has been suggested to reside in their ability to compete for cytoplasmic estrogen-binding sites or receptor, thereby reducing the formation of estrogen and estrogen-receptor complexes (8).

On the contrary, the steroidal antiestrogen, epitiostanol, has multiple hormonal and antihormonal activities such as androgenic, myogenic, progestational, antiestrogenic, and antigonadotropic activities but no demonstrable estrogenic activity (9,10). Furthermore, this compound shows a binding affinity to the estradiol receptors in the uterus and it acts as a typical competitive inhibitor on the cytoplasmic estradiol receptor in rat uterus (11).

When mepitiostane, a labile 17-ether substituent of epitiostanol, is given orally, it is incorporated into blood and estrogen-sensitive tissue; then it is gradually hydrolyzed and gives a sufficient amount of epitiostanol which is considered to be the active form of mepitiostane.

The regression rate and duration of regression obtained in the present study are very similar to those produced by tamoxifen and nafoxidin in patients with advanced breast cancer (12). The regression rate in the osseous group is higher than in the other groups who were administered these two nonsteroidal antiestrogens (12). This tendency is similarly noted in patients treated with epitiostanol (1) and might be essential to this kind of steroidal antiestrogen with moderate androgenic activity (10).

The side effects observed in this study were not serious and included virilizing effects such as hoarseness, hirsutism, and acne which were similar to those caused by the oral androgen, fluoxymesterone (13). No signs of drug-induced hepatotoxicity or hypercalcemia were found during treatment of these 45 patients. The drug could be administered in a patient with cirrhosis of the liver without any signs of acute elevation in the liver function test.

Further studies are needed to clarify any difference of efficacy in breast cancer between a nonsteroidal and this kind of steroidal antiestrogen.

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BCNU-Induced Perturbations in the Cell Cycle of 9L Rat Brain Tumor Cells[1,2,3,4]

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SUMMARY

Kinetic changes induced by BCNU in cultured 9L rat brain tumor cells were studied by means of conventional autoradiography and flow cytometry. Cell cycle parameters for the untreated cell line were: G_1, 8.5 hours; S, 8.2 hours; G_2, 3.2 hours; M, 0.5 hours; and cell cycle time, 19.5–20 hours. BCNU (5 μg/ml), which resulted in 99% cell kill as measured by the colony-forming efficiency assay, had the following effects: (*a*) undisturbed progression of G_2 cells toward mitosis; (*b*) prolongation of DNA synthesis time (from 8.5 to 13.7 hours); (*c*) moderate delay in the transition of cells moving from S to G_2; (*d*) progression of cells from early G_1 to G_2 but no division; (*e*) accumulation of the remaining cells in G_2 or M phase after one cell division; and (*f*) appearance of many nondisjunctive mitoses in cells in late G_1. Doses equal to 1 μg/ml of BCNU (20% cell kill) had little effect on proliferation and only moderately prolonged the S phase (10.5 hours). At this lower concentration, some cells remained arrested in the G_2 phase, but this phenomenon was much less extensive than it was at the higher BCNU concentrations. These data, obtained by sampling at many time points after BCNU treatment, may be helpful in interpreting the effects of BCNU on in vivo tumor systems which are much more complicated to analyze than in vitro models.

[Cancer Treat Rep 62:747–754, 1978]

The nitrosourea derivative, BCNU, has been utilized extensively in the chemotherapy of human malignant brain tumors (1,2). BCNU is lipophilic, crosses the blood-brain barrier, and distributes equally in tumor and adjacent normal brain tissue (3,4). This potent alkylator of nucleic acids is cell cycle nonspecific, a distinct advantage in treating tumors that have low growth fractions (5), and it has been evaluated in trials that involved both in vitro and in vivo brain tumor models (5–8). Rosenblum et al, using the 9L rat brain tumor model, have shown that a 0.75 or 1.0 × LD10 (toxic to 10% of the animals) dose of BCNU produces lifespan increases of 50%–100% and a 3-log cell kill, as measured by the colony-forming efficiency assay (7,8).

Although BCNU is one of the most effective single agents currently employed in brain tumor chemotherapy, it offers little hope by itself for tumor cure. As with most malignant tumors, factors such as the deleterious effects on the host's immune system, the heterogeneity of the tumor population, and the potential for development of drug resistance prohibit cure even when a 3-log cell kill can be achieved. Combination chemotherapy or multimo-

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dality therapy, spaced for maximum therapeutic effect, has been suggested as the solution to this dilemma.

Therapy involving BCNU as one component has been used in clinical trials, but the selection of complementary drugs and the timing of drug administration have been based on trial and error or the clinician's empirical decision. Obviously, treatment with one anticancer agent will create a perturbed tumor cell population that represents an entirely uncharacterized substrate for a second chemotherapeutic agent. The extent of the apparent synchronization and recruitment of G_0 cells into the proliferating pool, caused by perturbations such as cell death or accumulation in various phases of the cell cycle, must be determined for all drugs used in chemotherapy protocols. In the study described here, autoradiography and flow microfluorometry were used to analyze the effects of BCNU on the cell kinetics of cultured 9L rat brain tumor cells.

MATERIALS AND METHODS

Cell Line

The cells used in this study were derived from a brain tumor (9L) originally induced in a CD Fischer rat by 1-methyl-1-nitrosourea (6,9), and were cultured as monolayers at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was Eagle's Basal Medium with 10% fetal calf serum and antibiotics (penicillin, 80.5 units; streptomycin, 80.5 μg/ml); all components of the medium were obtained from Microbiological Associates Inc (Bethesda, Md). The cell cycle parameters for this cell line, as determined from the percent labeled mitoses (PLM) curve following a pulse of ^{3}H-labeled thymidine (^{3}H-TdR), were: G_1, 8.5 hours; S, 8.2 hours; G_2, 3.2 hours; M, 0.5 hour; and cell cycle time, 19.5–20 hours. The growth fraction, determined by continuous incubation with ^{3}H-TdR over an entire cell cycle time, was approximately 1 (10). Unless otherwise stated, the following protocol was used to treat 9L cells in culture: (*a*) 5 × 10^5 cells were seeded in 75-cm^2 Falcon tissue culture flasks (Falcon Plastics, Oxnard, Calif) containing 10.0 ml of medium; (*b*) cells were allowed to remain undisturbed for 48 hours to provide an asynchronous, exponentially growing culture for treatment with BCNU. A modified cell counter (Royco Instruments, Inc, Menlo Park, Calif) was used for cell counts. BCNU was stored at –79°C. To prepare the various stock BCNU concentrations, the drug was weighed out and immediately dissolved in absolute ethanol; aliquots from this solution were then diluted in varying amounts of calcium- and magnesium-free Hanks' balanced salt solution (GIBCO, Grand Island, NY) to obtain the various stock concentrations (11). Immediately after dilution, 1 ml of stock BCNU was added to the cells growing in treatment flasks, to provide the desired final BCNU concentration.

Wheeler and co-workers (11) of this laboratory have shown that BCNU was completely broken down in 2 hours under experimental conditions similar to those used in our study and that extracellular breakdown products of BCNU had no effect on the subsequent survival of cells. Since the removal of such products was not necessary, any perturbations to the cells that might be caused by washing and the addition of fresh medium were avoided in this study. Under these conditions, the percent survival of 9L cells treated with 1 and 5 μg/ml of BCNU for > 2 hours was 80% or 1%, respectively (11).

Effect of BCNU on Growth Rate

Cultures containing 5 × 10^5 cells initially were treated according to the following schedules: (*a*) medium alone; (*b*) medium containing 1 μg/ml of BCNU; and (*c*) medium containing 5 μg/ml of BCNU. On each of 5 successive days after BCNU treatment, three cultures from each experimental group were trypsinized (0.25% trypsin [GIBCO] + 20 mg/100 ml EDTA [University of California Pharmacy]) at 37°C for 5 minutes and the number of cells in each sample was counted. Kinetic parameters were estimated by several techniques.

Autoradiographic Method

Pulse chase method

Each flask containing 10 ml of medium received 1 microcurie of ^{3}H-TdR (specific activity, 0.36 curie/mmol; Schwartz Bio-Research, Inc, Orangeburg, NY); these cultures, which initially contained 5 × 10^5 cells, were incubated for 30 minutes and then washed twice with cold calcium- and magnesium-free Hanks' balanced salt solution. Fresh medium, pre-equilibrated to 37°C and pH 7.3, containing either 1 or 5 μg/ml of BCNU diluted just before treatment, was added to the culture. During the next 46.5 hours of incubation, three replicate flasks were trypsinized every 1.5 hours. The medium from each culture, containing detached single cells, was combined in the same test tube with the trypsinized cells from each flask to insure that all cells from a given sample would be accounted for (12). During such procedures, it is essential to include mitotic cells, which have a tendency to become spherical and detach from the glass. After centrifugation (221

× g, 4°C, 5 minutes), the cells were treated for 5 minutes with a hypotonic solution (2.8 g NaCl/liter of 0.002 M phosphate buffer at pH 7.0). After recentrifugation, the cells were fixed (absolute ethanol:acetic acid, 3:1) for 5 minutes and spread on a microscope slide (12). The slides were air-dried, dipped in Kodak NTB-2 nuclear emulsion (Kodak, Rochester, NY), and exposed at 4°C in light-tight boxes containing Drierite (W.A. Hammond Co, Xenia, Ohio) for 10–14 days. The autoradiographs were developed in Kodak D-19 for 4 minutes at 18°C, fixed in Kodak Rapid Fixer, rinsed, dehydrated, stained with hematoxylin, and mounted for light microscopy. A PLM curve was constructed after 50–100 mitoses were examined on each slide. The mitotic index (MI) (percentage of cells undergoing mitosis) and labeled population (LP) (percentage of cells labeled with ^{3}H-TdR) were determined by scoring 1000–3000 cells from identical slides. "Labeled" cells were defined as those containing $>$ 4 grains/nucleus. A grain index was determined by counting grains on 100 nuclei on each slide prepared from six specimens harvested at 0 and 41.5 hours.

Labeling index (LI)

Approximately 50 cultures, each seeded with 5×10^5 cells in 10 ml of medium, were incubated for 48 hours and then mixed with 1.0 ml of 55 μg/ml of BCNU solution, for a final concentration of 5 μg/ml. After administration of BCNU, 1.0 microcurie of ^{3}H-TdR was added at various times (1, 3, 6, 9, and 12 hours, then every 4–48 hours) to three of these cultures. After a 15-minute incubation with ^{3}H-TdR, cells were washed twice with cold calcium- and magnesium-free Hanks' balanced salt solution. The harvested cells were fixed with acetic acid/alcohol and prepared for autoradiography. The mean LI (percentage of cells that incorporated ^{3}H-TdR during a 15-minute pulse-labeling exposure at various times after BCNU treatment) with standard deviation was determined from the number of ^{3}H-labeled cells in the population ($>$ 1000 cells scored).

Flow Cytometric (FCM) Analysis

One random sample from cultures prepared for the pulse chase study was harvested every 3 hours by trypsinization and fixed in 10% buffered formalin. The cellular DNA was hydrolyzed with 4 N HCl at ambient temperature for 20 minutes before the cells were stained with acriflavin (0.02 g acriflavin + 0.5 g $K_2S_2O_5$ in 10 ml of 0.5 N HCl and 90 ml of distilled water) for another 20 minutes as previously described (13). These specimens were analyzed on the flow cytometer at the Lawrence-Livermore Laboratories of the University of California (14,15).

RESULTS

Growth Rate

The comparative growth curves for 9L cells in the presence and absence of BCNU are shown in figure 1. Cultures maintained in standard medium grew exponentially to approximately 5×10^6 cells/flask in 5 days, after which the growth rate decreased. The effect of BCNU on cell growth varied from mild retardation at a final concentration of 1 μg/ml to significant cell kill at 5 μg/ml. During the first day of treatment with 1 μg/ml of BCNU, there was an initial increase in cell count from 6.6×10^5 to 1.75×10^6, after which the population increased at a relatively slow rate for 2 days and then decreased. In the presence of 5 μg/ml of BCNU, only an initial population increase was noted, followed by progressive depletion of the cell population due to either lysis or destruction of dead cells during the harvesting procedures.

Autoradiography

Pulse chase method

A. Effects of 1 μg/ml of BCNU.—The PLM curve (fig 2B) revealed an appreciable increase in labeled mitoses beginning 3.5 hours after the addition of BCNU; this period may correspond to the duration of the G_2 phase. The PLM reached nearly 100% 7 hours after administration of BCNU and then decreased gradually to 15%. The duration of S phase, calculated by measuring the interval between the ascending arm and the descending arm of the PLM curve at the 50% level, was 10.5 hours. Analysis of the MI revealed a gradual decrease to 50% of the control value by 17 hours; the MI remained at this level for 10 hours before increasing again. The LP of 36% ± 2.5% (± SD) at 0 time increased to 53% ± 2.3% at 17 hours, before decreasing to 39% ± 5.0% at 35 hours after treatment.

B. Effects of 5 μg/ml of BCNU.—The PLM curve is depicted in figure 2C. It was noted that the first wave is wider, and both ascending and descending arms of the wave are less steep compared to the PLM curve obtained from the 1-μg/ml BCNU-treated cells (fig 2B). However, there was no appreciable change in the time of initial appearance of labeled mitoses, and no second wave of labeled mitoses was observed. The transition times of S and G_2 phases were 13.7 and 3.5 hours, respectively. A gradual decrease of MI occurred between 5 and 15 hours. This nadir was maintained for 15 hours and then was followed by an increase of MI beginning at 30 hours (fig 2D). Examination of the LP in this experiment revealed that the initial LP of 40.9% ± 2.2% increased to 51.8% ± 2.4% in 13.5 hours, then gradually decreased to 45.5% ± 2.3% beginning 20 hours thereafter (fig 3). Morphologic examination of these slides revealed that bridging of chromosomes or nondisjunctive mitotic figures and cells with highly fragmented nuclei appeared 19–20 hours after the treatment and increased in number thereafter. It is of particular interest that most of these abnormal cells had not incorporated any ^{3}H-TdR (fig 4). The mean grain number per nucleus was 18.8 ± 10.1 at 0 time and 8.0 ± 4.2 at 41.5 hours.

Labeling Index

The LI was followed for 48 hours after treatment with BCNU (fig 5). The data indicated that the percentage of cells engaged in DNA synthesis was almost constant over a 36-hour period (LI range = 35%–41%). This percentage abruptly decreased to < 10% at 48 hours, while the LI of control cultures remained in the range of 35%–41% during this entire period.

FCM Analysis

Representative FCM profiles of BCNU-treated 9L cells are shown in figure 6. The profile of untreated cells (0 hour, fig 6) exhibits a large peak on the left, representing cells that possess a 2C (G_1) DNA content. The smaller peak at the right represents

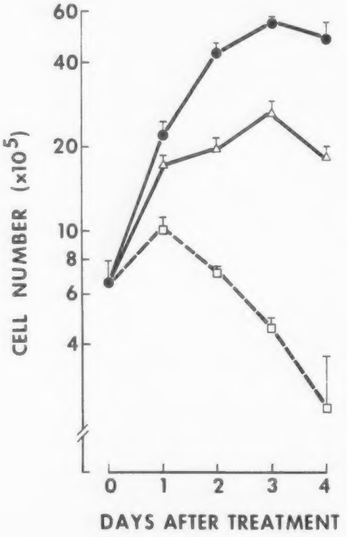

FIGURE 1.—Comparative growth curves for 9L cells treated with BCNU. ● = controls; △ = 1 μg/ml of BCNU; □ = 5 μg/ml of BCNU. Each point indicates the mean No. of cells calculated from 3 flasks. Bars at each point = SD.

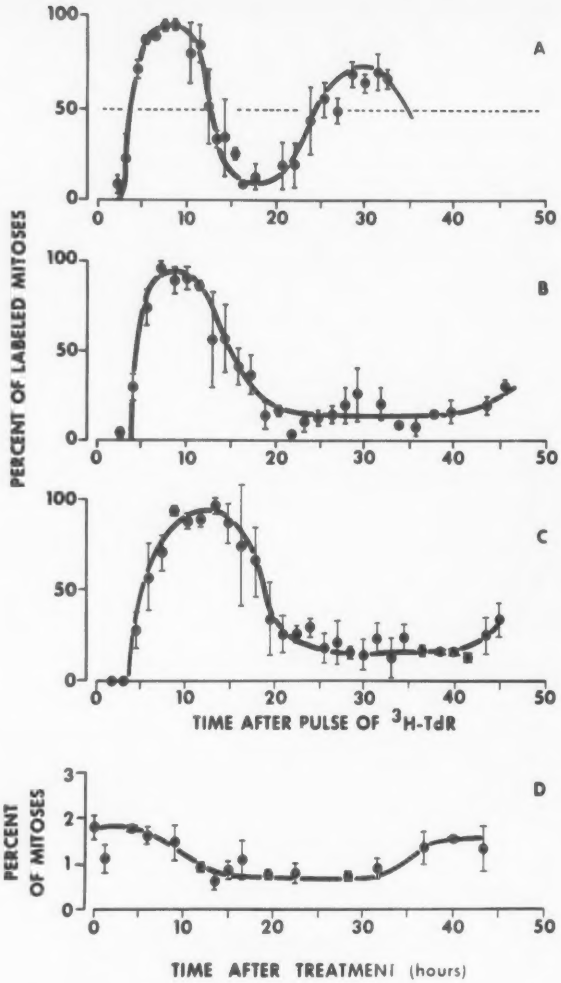

FIGURE 2.—PLM of (A) unperturbed 9L cells, and cells treated with (B) 1 μg/ml of BCNU and (C) 5 μg/ml of BCNU. (D) % MI of 5-μg/ml BCNU-treated cells. Bars at each point = 1 SD.

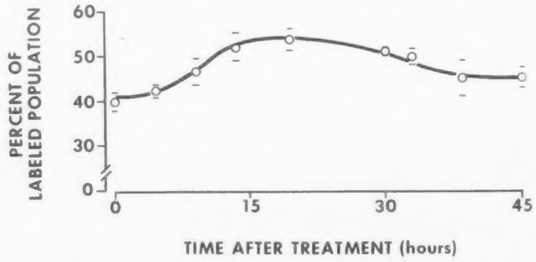

FIGURE 3.—Analysis of changes in ^{3}H-labeled population after 5 μg/ml of BCNU. Labeled population means a fraction of cells labeled at various times after a 30-min exposure to ^{3}H-TdR. Bars at each point = SD.

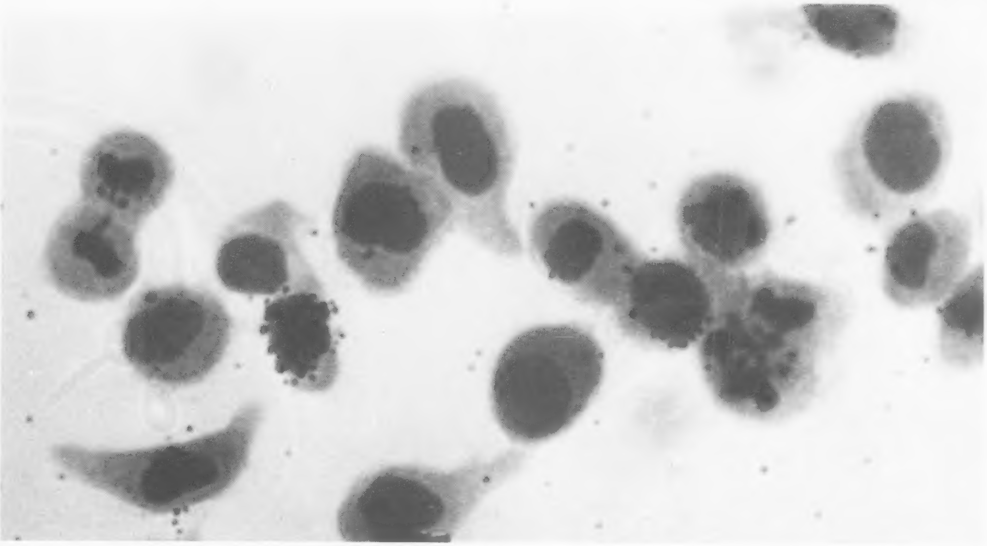

FIGURE 4.—Bridging of chromosomes (left) and cells with highly fragmented nuclei in mitosis (right); most of these abnormal cells were not labeled with ^{3}H-TdR.

cells with a 4C DNA content, an indication that these cells are in G_2 or M phase. The interval between the two peaks results from cells in S phase that have synthesized varying amounts of DNA during their progression through this phase. Cells that register near the 4C peak have replicated nearly a full complement of DNA and are in late S phase, while those close to the 2C peak are in early S phase. BCNU treatment resulted in little alteration of the profile for subsequent 2-hour intervals, but the number of cells in early S phase decreased gradually over the next 4–12 hours. After 24 hours, the depleted early-S phase population was augmented by cells passing through the cycle for the second time. After 36 hours, many cells were located in late interphase, and virtually all cells were in this phase after 48 hours. In addition, after 48 hours a significant number of cells produced a third peak at the 8C position, indicating a DNA content greater than that at the 4C position.

DISCUSSION

The initial increase in cell count that we observed during the first day of treatment with 1 μg/ml of BCNU indicates that the cells can continue to divide for about 24 hours in a relatively undisturbed manner (fig 1). The population increase during the first day in the presence of 5 μg/ml of BCNU shows that some cells were either killed or blocked in one phase of the cell cycle, but that at this level of BCNU many cells were able to undergo cell division. Because the most striking kinetic changes occurred in cells treated with 5 μg/ml of BCNU, our discussion will focus on the results obtained at this drug concentration.

The major changes in the behavior of 9L rat brain tumor cells exposed to 5 μg/ml of BCNU may be summarized as follows:

1. there is undisturbed progression of G_2 cells toward mitosis;
2. DNA synthesis time is prolonged;
3. there is a delay in the transition from S to G_2;

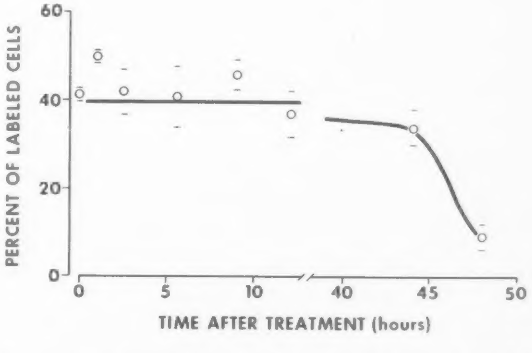

FIGURE 5.—Followup of LI after 5 μg/ml of BCNU. % labeled cells demonstrates a fraction of cells labeled immediately after a 15-min exposure to ^{3}H-TdR administered at various times following BCNU treatment. Bars at each point = 1 SD.

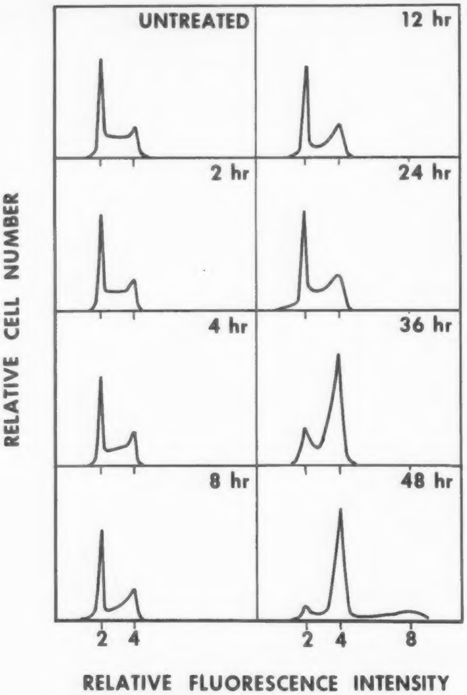

FIGURE 6.—FCM analysis of 9L cells treated with 5 μg/ml of BCNU. Relative fluorescence intensity values of 1.0 and 2.0 correspond to the position of nuclei with 2C and 4C DNA contents, respectively.

4. cells in early G_1 progress to G_2 but do not divide;
5. remaining cells accumulate in G_2 or M after one cell cycle; and
6. cells in late G_1 at the time of BCNU treatment show more nondisjunctive mitoses.

We did not observe a direct effect of BCNU on cells that were in G_2 at the time of treatment; the time of the appearance of labeled mitoses was the same for treated and control cultures. This is in contrast to the results of Barranco and Humphrey (17) which indicated that BCNU caused some perturbation of CHO cells that were in G_2 at the time of drug administration. In addition, there was no change in MI during the first 5 hours after exposure to BCNU. PLM analysis for estimation of S phase required that all cells in S phase initially entered the mitotic phase. This was confirmed by the grain index, which showed 8.0 ± 4.2/nucleus at 41.5 hours in contrast to 18.8 ± 10.1 at 0 time after treatment. This finding suggests that the daughter cells shared the ^{3}H-labeled DNA equally. Then the estimated S phase was prolonged by 5.5 hours (67%), from 8.2 to 13.7 hours, when cells were incubated in 5 μg/ml of BCNU. Reports based on both in vitro (16–18) and in vivo (19–21) studies have provided evidence that nitrosoureas induce a prolongation of the DNA synthetic phase; in contrast, classic alkylating agents produce little S-phase extension (22,23). The decreasing slope of both arms of the PLM curve may reflect a temporary blocking effect at the S-G_2 and G_1–S boundaries. This delay in progression was also observed in other cell systems (17). BCNU did not cause late G_1 cells to become arrested in the G_2 phase; this is demonstrated by a decrease in the first peak of the PLM curve due to dilution by nonlabeled late G_1 cells that had undergone mitosis. The LP (fig 3) gradually rose from 40.9% to 51.8% in the first 13.5 hours, due to multiplication of labeled cells ($P < 0.001$). This increase (51.8%) was then diminished to 47.5% ($P < 0.05$) beginning 20 hours after treatment, due to the multiplication of the unlabeled G_1 cells. Since this dilution of the LP (to 47.5%) did not lower the LP to the original level of 40.9% ($P < 0.005$), it must be assumed that not all of the unlabeled G_1 cells were capable of completing mitosis. This interpretation is supported by cinephotomicrographic analysis of 13 early G_1 cells. Of 24 cells studied, it was observed that the 13 cells which were unable to divide after BCNU treatment had been exposed to the drug during the 6 hours following their last complete mitosis. These cells did not divide during an 82-hour period after 5 μg/ml of BCNU treatment (U. Ehmann, personal communication). Our observation that there was only a 60% increase in cell number, rather than a doubling of the population of the 5-μg/ml BCNU-treated cells (fig 1), could be explained by the death of arrested cells, while cells not arrested were undergoing cell division.

The absence of reasonable second peaks in the PLM curve indicates that cells in late G_1, S, and G_2 could complete mitosis but were not capable of entering a second mitotic phase. The LI of 38% over the initial 44-hour period (range, 35%–41%) after treatment and its abrupt decrease to < 10% at 48 hours also suggest that most cells could progress into S phase of the second cycle before becoming arrested somewhere between S and G_2 of the second cycle (fig 5). Regardless of whether these cells completed one cell cycle or not, the FCM profiles suggest that they were arrested with a 4C DNA content within the observation period of 48 hours. If these cells with a 4C DNA content were arrested at G_2, the number of mitotic figures should have decreased shortly after all the cells from late G_1 completed cell division. However, the MI gradually returned to the normal value by 30 hours after drug treatment.

Perhaps nondisjunctive mitoses and/or mitotic arrest were responsible for the discrepancy between the apparent recovery of MI in the absence of an increase in cell number. The increased chromosome bridging with absence of labeling that was observed beginning 20 hours after treatment suggests that some cells in late G_1 are particularly susceptible to drug-induced aberrations in this phase. BCNU, then, may arrest cells according to two schedules: (*a*) some cells could divide at least once before arrest at G_2, and (*b*) other cells could be arrested at G_2 initially. The latter category consists of cells in early G_1 at the time of treatment. Therefore BCNU-induced DNA damage in early G_1 cells might be different from the damage induced in cells that were in other phases of the cell cycle when BCNU was added to the cell cultures.

According to our data, only cells treated with BCNU in early G_1 are prevented from further divisions, even though DNA synthesis occurs. One possible explanation is that interstrand crosslinking of DNA is responsible for this inhibition of mitosis (24). In addition, Hilton (personal communication, 1978) reports that in a recent in vivo study of 9L cells implanted in rat brain, DNA-DNA crosslinking (as measured by a modified alkaline elution technique) existed up to 36 hours after ip administration of BCNU. Interstrand crosslinking is possible between two mature strands of DNA (parent-daughter). Intrastrand crosslinking is also possible, both in mature DNA and newly replicated DNA, but probably has little or no effect on cell division. If interstrand crosslinking between parent DNA strands is prevented in cells that are in S and G_2 phase, because of structural or biochemical factors, then the observed phenomena could be explained as follows. Those cells in early G_1 at the time of BCNU treatment might not be able to divide because of interstrand crosslinking between the two parent DNA molecules. For cells in S and G_2, interstrand DNA crosslinking would exist within a semiconservatively replicated DNA hybrid. Since the two parent DNA molecules are not crosslinked, the two sets of semiconservative DNA are free to separate and the first cell division can be completed. However, the two DNA strands in the newly formed daughter cells are already crosslinked and cannot separate during a subsequent mitosis, although they may serve as templates for the synthesis of new DNA.

The sensitivity of cells in G_0 to BCNU has been demonstrated by the in vivo cell kill of 3 logs in the same line implanted ic into rats; 50% of this tumor consists of noncycling cells (7). These observations indicate that the sensitivity of G_0 cells to BCNU may be related to the mechanism that controls the sensitivity of early G_1 cells, since G_0 cells could be considered similar to early G_1 cells in terms of DNA content and biologic characteristics. Although we do not yet understand how BCNU creates blocks in the cell cycle, Rao and Rao (25) have reported that CHO cells treated with BCNU and other drugs accumulate in G_2 because of their failure to progress into mitosis as a direct or indirect result of extensive chromosome damage. From the chemotherapeutic standpoint, it is important to determine where in the cell cycle the surviving cells from an in vitro treatment accumulate.

Tobey and Crissman described in detail the effects of three nitrosourea analogs, including BCNU, on survival and cell cycle traverse capacity in exponentially growing populations of CHO cells and in isoleucine-deprived cells arrested in G_1 (16). They showed that cells treated with nitrosourea analogs accumulated in late S and G_2; however, they examined only cells that had undergone a 30-hour incubation after a 2-hour exposure to BCNU. Their results suggest that a large fraction of cells could divide once (or possibly twice) in cycling populations, based on the increase in cell number observed after a 30-hour period subsequent to drug removal after the initial 2-hour exposure period. These data are in good agreement with the results obtained by us. Tobey and Crissman also reported that synchronized cells in early G_1 showed prolongation of S phase but no effect on traverse of G_1 and initiation of DNA synthesis when cells were treated with 10 μg/ml of methyl-CCNU.

Tobey and Crissman have suggested that maximum cell kill might be achieved by administration of a G_2- and/or M-specific cytotoxic agent only after an interval corresponding to maximum accumulation in G_2. In our experiments, treatment with 5 μg/ml of BCNU resulted in 99% cell kill under such conditions, and most of the cells arrested in G_2/M phases eventually died. Therefore, the suggestion offered by Tobey and Crissman would apply only when surviving cells are blocked in the G_2 phase. However, if the surviving cells are distributed in any phases of the cell cycle other than in G_2 or M, a different mode or combination of therapies will be required to achieve maximum cell kill. It is very difficult to extrapolate from in vitro experiments to the in vivo situation. However, our experiments have shown, at the least, that some cells in the proliferating pool can escape from the action of BCNU. Because an LD10 dose of BCNU to 9L tumor-bearing rats caused a 3-log cell kill (7,8) and since the surviving cells will not always originate in the nonproliferative pool, it may be that the use of a *combination* of cell cycle specific drugs will increase overall cell kill. In this possibility lies the significance of our studies for improving the design of future clinical chemotherapy trials.

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Rate of DNA Synthesis as an Indication of Drug Toxicity and as a Guide for Scheduling Cancer Therapy [1,2]

Glynn P. Wheeler* and Jo Ann Alexander [3,4]

SUMMARY

The rates of incorporation of [C^3H_3]thymidine into the DNA of tissues of normal mice at various times after single doses of several drugs (adriamycin, vincristine, methotrexate, 5-fluorouracil [5-FU], melphalan, CCNU, and chlorozotocin) that have some clinical activity against cancer have been determined and are considered to be indicative of the "degree of normalcy" of the respective tissues at those times. These experiments show that there are some differences and some similarities in the effects of the agents upon the various tissues of the mouse. Each of the agents caused early inhibition of incorporation of [C^3H_3]thymidine followed by stimulation of such incorporation by intestinal mucosa and marrow, but the timing and the extents of inhibition and stimulation differed for the various agents. Except for CCNU, the preceding description would also apply to the effects upon liver and lungs, but the stimulation was delayed in comparison to that of intestinal mucosa and marrow. Early inhibition with little subsequent stimulation occurred in the spleen, kidneys, and heart. For all tissues, recovery was slower and less extensive after CCNU than after the other agents. The data obtained with the single agents were used for setting up experiments in which a single dose of one agent was followed after selected intervals of time with a single dose of a second agent. The combination of adriamycin plus 5-FU was less toxic when the two agents were given simultaneously than when the 5-FU was given 24–96 hours after the adriamycin, while for the combination of adriamycin plus CCNU there was less toxicity if the CCNU was administered 24 hours after the adriamycin rather than simultaneously with it. When a combination of a single dose of methotrexate and a single dose of 5-FU was administered to mice, the lethality was greater when the two agents were administered 120 hours apart than when the two agents were given simultaneously or were separated by 24 or 48 hours; this was true regardless of which agent was administered first. These results are consistent with what might be predicted upon the basis of the data obtained for the single agents in this study and of what is known about the mechanisms of action of these agents and of the effects of the agents in relation to cell and tissue kinetics. These results indicate that patterns of thymidine fixation by tissues following single doses of agents might be useful in scheduling multiple-drug therapy to minimize host toxicity.

[Cancer Treat Rep 62:755–769, 1978]

It has been pointed out (1) that oncologists have developed the following broad guidelines for consideration in designing combination-chemotherapy regimens: "(*a*) each drug included should be active when used alone against the neoplasm in question; (*b*) the drugs should have different mechanisms of action; and (*c*) the toxic effects of the drugs should not overlap so that each can be given at or near its maximum tolerated dose." Other chemotherapists might also add that there should be no evidence of cross-resistance to the agents used in the combina-

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tion and that the relevance of tissue and cell kinetics to the effects of the agents of the combination must be considered. The present report relates to (*c*) and concerns the toxicities of individual agents for various tissues of the mouse, since it is desirable to know the effects of the individual agents to serve as background information for experiments with combinations of agents.

The toxic effects of therapeutic agents are usually recognized by gross or microscopic examination of the tissues, by altered peripheral blood counts, by altered levels of enzymes in serum, by altered levels of metabolites in blood and urine, and by gross effects, such as alopecia, dermatitis, stomatitis, etc. A number of these methods are rather subjective, and, since different methods are used for different tissues, comparison of the effects upon different tissues is difficult. Therefore, it is desirable to have a method that will show the effects of an agent upon a variety of tissues and that can be used to study the effects of anticancer agents with a variety of mechanisms of action.

If it is assumed that all tissues (with the possible exception of nerve tissue) of the adult animal are in a dynamic state involving cell death, cell loss, and cell replacement, then there would be cell proliferation and synthesis of DNA occurring continuously to some extent in all of these tissues. In normal healthy animals it would be expected that the rates of cell proliferation and of synthesis of DNA would fall within a "normal" range for each tissue. It might also be expected that a "damaged" tissue would have an altered rate of cell proliferation and synthesis of DNA. Therefore, the rate of synthesis of DNA would be an indication of the "degree of normalcy" of the tissue, and the extent of alteration of the rate would be a measure of the extent of the damage. With certain exceptions (eg, when an agent causes a change in the sizes of the pools of phosphates of thymidine), the rate of incorporation of [C^3H_3]thymidine into DNA is a satisfactory measure of the rate of synthesis of DNA by the cell. It was recently concluded (2) that the rate of incorporation of [C^3H_3]thymidine into DNA was as satisfactory as the conventional radioautographic technique of determining labeling index or the functional kinetic technique of granulocyte colony formation in vitro for studying the injury induced by antitumor agents to normal and cancerous tissues. In the present study, the incorporation in vivo of [C^3H_3]thymidine into the DNA of a variety of tissues of normal, non-tumor-bearing mice and of non-tumor-bearing mice that had received single injections of specified anticancer agents was used to determine the "degree of normalcy" of the tissues.

Several groups of investigators have used essentially this same method to compare the effects of single agents upon the rates of incorporation of [C^3H_3]thymidine into the DNA of transplanted ascitic or solid cancers and the intestinal mucosa and marrow of the hosts. We have previously reported two studies of this type (3,4), and Young (5) has used this method rather extensively. Among the agents that have been studied are cytosine arabinoside (6–8), methotrexate (5,9), 5-fluorouracil (5-FU) (5,10,11), 5-azacytidine (8), cyclophosphamide (2,4,5,12,13), nitrosoureas (3,7,12,14–18), and adriamycin (19,20). Since a variety of test systems and procedures were used in these previous studies and in most of them only two or three tissues were examined, it seemed worthwhile to compare several agents in a single test system and to examine more tissues. In the present investigation, we have determined the magnitude and the duration of the effects of several agents upon the incorporation of [C^3H_3]thymidine into the DNA of intestinal mucosa, marrow, spleen, liver, kidneys, heart, and lungs of mice. Several experiments were also performed to demonstrate how these data can be used to plan schedules for two-drug chemotherapy.

Although some experiments were performed with cancer-bearing mice, the initial experiments to obtain base data were performed with normal mice, since previous experiments (3) have shown that the presence of a cancer in an animal can cause alteration in the metabolic activities of the host tissues, and such alterations complicate the interpretation of the data.

METHODS

Non-tumor-bearing adult $B6C3F_1$ (C57BL/6J × C3H) mice in groups of five animals received single doses of the specified agents ip at dosage levels of LD10/2 and LD10. At various time intervals thereafter the mice were given ip injections of [C^3H_3]thymidine (New England Nuclear Corp, Boston, Mass; diluted to 37.7 millicuries/mmol) at a level of 1 microcurie/g of body weight, and the mice were killed 2 hours later by carbon dioxide asphyxiation. The livers, lungs, hearts, kidneys, and spleens were dissected from the five mice of each group and pooled in ice-cold beakers. The marrow was forced from the femurs with a solution containing sodium *p*-aminosalicylate (6%, wt/vol) and sodium dodecyl sulfate (1%, wt/vol) by means of a syringe attached to the bone by plastic tubing, and the suspensions of marrow from the five mice were pooled. The contents of the excised intestines were expressed by applying pressure with a scalpel, and the intestines were then slit and the mucosa were removed by scraping with a scalpel and pooled. DNA was isolated from the tissues by the method of Frei and Lawley (21), except that the procedure was stopped after the first precipitation of the DNA which was washed with ethanol and ether and dried. Portions of the dry DNA were dissolved in water, and samples of this solution were used for the determination of DNA content by the method of Schneider (22) and for radioassay with a Packard Model 3315 liquid scintillation spectrometer.

In two experiments, mice of the above strain bearing subcutaneous 14-day-old C3H-16/C mammary adenocarcinomas were used, and the tumors and host tissues were studied by the methods described above. This transplantable tumor was developed at the Southern Research Institute, Birmingham, Ala, by Dr. T. H. Corbett and co-workers, and a manuscript describing its properties and giving its responses to a number of anticancer agents is in preparation.

The nominal ip LD10 doses of the agents, which were determined previously by the Chemotherapy Department of this Institute and used in these experiments, were: adriamycin (15 mg/kg); vincristine (2.6 mg/kg); methotrexate (100 mg/kg); 5-FU (180 mg/kg); melphalan (15 mg/kg); CCNU (50 mg/kg); and chlorozotocin (20 mg/kg). However, in the toxicity experiments that are presented in this report, adriamycin at this dosage killed $> 10\%$ of the animals, and CCNU at the indicated dosage killed $< 10\%$.

For the determination of the effects of two-drug combinations upon the lifespan of mice when the agents were administered in various time sequences, the agents were injected ip at the dosages given above into groups of randomly selected animals, and the groups were checked daily for dead animals. Data for the individual schedules are given in the Results section, where the particular experiments are described.

RESULTS AND DISCUSSION

It is emphasized that in the present study the measurement of the incorporation of thymidine into the DNA is used to determine the "state of health" or "degree of normalcy" of the tissues and not to determine the mechanism of action of the agent. Nevertheless, in interpreting the experimental results it is necessary to keep in mind what is known about the mechanisms of action of the agents and to consider the possible causes of the observed decrease or stimulation of incorporation of [C^3H_3]thymidine into the DNA. Some possible reasons for decreased incorporation include: (*a*) inhibition of the synthesis of building blocks for the synthesis of DNA; (*b*) inhibition of the polymerization of deoxyribonucleoside triphosphates; (*c*) altered cell and tissue kinetics; and (*d*) presence of dead, nonmetabolizing cells in the tissues. Some possible reasons for the stimulation or "overshoot" of incorporation include: (*a*) triggering of stem cells or resting cells into proliferation; (*b*) removal of nonproliferating cells; and (*c*) decreased pools of endogenous thymidine phosphate and its precursors. It is also assumed that there will eventually be a return to the normal rate of incorporation for each tissue and that this return to normalcy is indicative of a recovery from the damage.

Table 1 contains the mean values and the standard deviations of the specific radioactivities of the DNA of tissues from control mice of 13–15 experiments. The experimental results for the incorporation of [C^3H_3]thymidine into DNA of the tissues of treated mice are presented graphically in a way that has been used previously (3). The differences of the specific radioactivities of the tissues of treated animals from the mean specific activities of the tissues of the untreated control animals were divided by the standard deviations for the respective control tissues, and the quotients were plotted *versus* the time after treatment that the [C^3H_3]thymidine was injected. The resulting curves show whether the values are less than or greater than the reference values, and the values on the ordinates serve as guides to indicate the statistical significance of the differences. Therefore, in a single graph it is possible to show the relative effects of the agents upon the incorporation of [C^3H_3]thymidine into the various tissues, although the absolute specific activities of the various tissues may differ greatly.

Effects of Doubling the Dose

Examination of the effects of doubling the doses of all of the agents from LD10/2 to LD10 upon all of the tissues showed that in 50% of the instances

TABLE 1.— Specific radioactivities of the DNA of tissues of control animals 2 hrs after the ip injection of [C^3H_3]thymidine

Tissue	picocuries/μg of DNA	
	Mean*	SD*
Intestinal mucosa	77.25	13.43
Marrow	79.30	17.52
Lungs	9.84	4.05
Liver	6.39	2.37
Spleen	63.73	16.69
Kidneys	3.83	1.20
Heart	4.92	2.06

*These values are based upon the data obtained in 13–15 separate experiments. In each experiment the tissues of 5 animals were pooled.

doubling the doses did not significantly alter the patterns of incorporation of [C^3H_3]thymidine. The most common effect of doubling the dose was an increase in the extent of the "overshoot," and in a number of instances the "overshoot" was delayed by several hours. Doubling the doses had relatively less effect upon the degree of inhibition of the incorporation, as shown by the levels of the nadirs of the curves. Therefore, only the data from the LD10 experiments are presented in this report.

Effects of Single Agents Upon Various Tissues

Data are given in figure 1 for the effects of LD10 doses of the agents upon the various tissues. Cursory scanning of the graphs leads to the recognition that the tissues fall into three groups based upon the observed effects of all of the agents upon the particular tissues.

1. *Intestinal mucosa and marrow*—all of the agents except methotrexate and vincristine in the case of mucosa and methotrexate, vincristine, and adriamycin in the case of marrow caused extensive inhibition by 24 hours, and there were "overshoots" by all agents by 48–96 hours. Some of the agents caused secondary inhibitions.
2. *Lungs and liver*—all of the agents caused inhibition in lungs at 24 hours with recoveries to normal by 48 hours for vincristine and methotrexate. All agents except vincristine and methotrexate caused inhibition in liver at 24–48 hours. With both tissues there were delayed and extended "overshoots" for most agents in comparison with "overshoots" for intestinal mucosa and marrow.
3. *Spleen, kidneys, and heart*—with all of the agents there was inhibition at 24 hours with slow recovery and little or no "overshoot" for most agents.

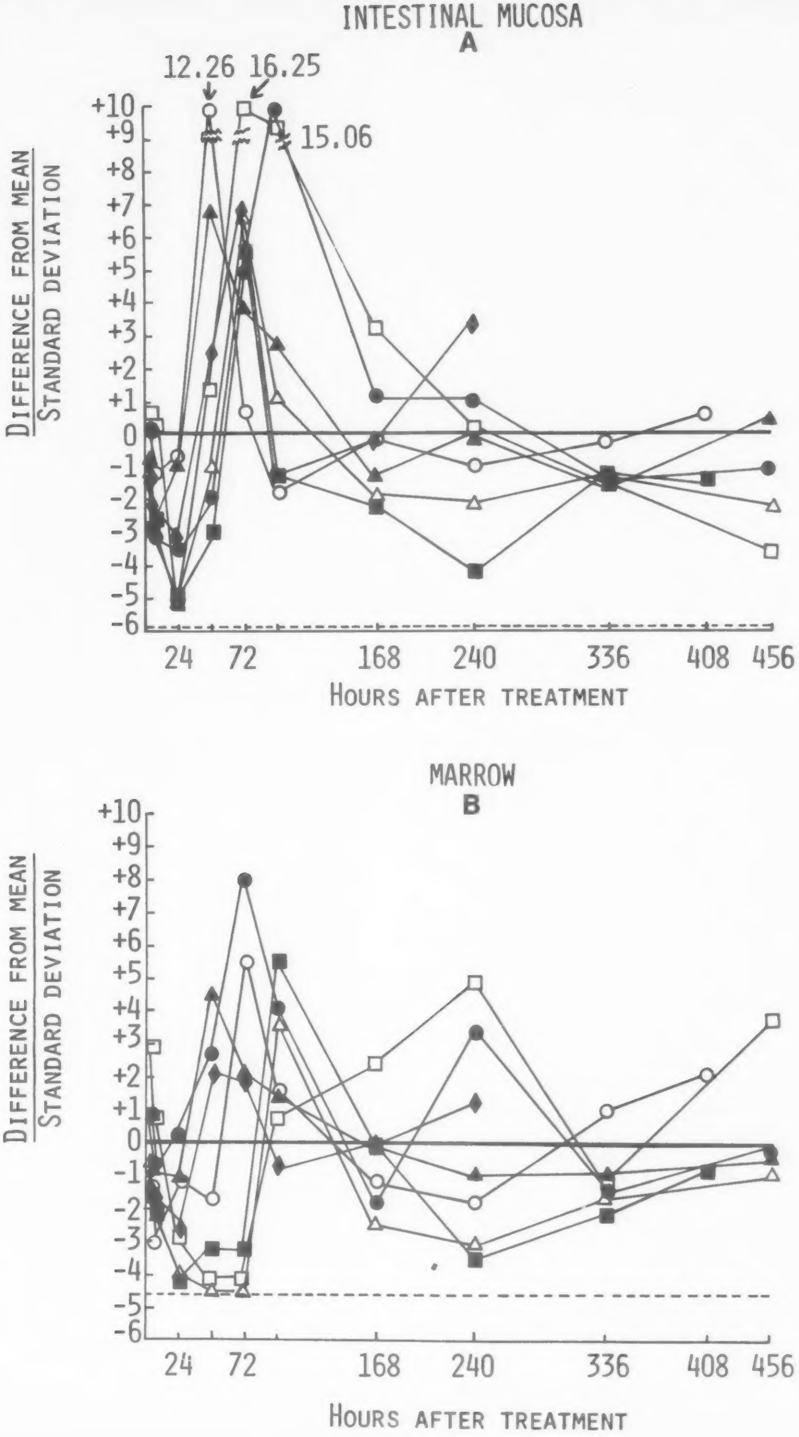
Although the above generalizations seem justified, the following specific effects of certain agents were noted.

1. Methotrexate and 5-FU caused delayed and extended "overshoots" for a number of tissues with peaks at 168–240 hours. It seems unlikely that these late increases in the incorporation of [C^3H_3]thymidine would be due to decreased endogenous pools of thymidine phosphates, although no data for the sizes of such pools at these times are available.
2. With CCNU there were "overshoots" only for intestinal mucosa and marrow, and there were secondary inhibitions with these tissues that were almost as great as the early inhibitions that occurred at 24 hours. These secondary inhibitions and the slow recoveries of the other tissues seem consistent with the delayed toxicity that has been reported for this agent. For most of the tissues CCNU was the most inhibitory of the agents tested.
3. The early effects of chlorozotocin were quite similar to those of CCNU, but all of the tissues recovered more readily and there was no extensive secondary inhibition of intestinal mucosa or marrow. These results are of interest in view of the report (18) that chlorozotocin has a lower toxicity to marrow than CCNU.
4. With melphalan the "overshoots" occurred only with intestinal mucosa, marrow, and lungs, and secondary inhibitions occurred with each of these tissues.

It is clear that all of the agents do not affect a specific tissue in the same way and that a single agent does not affect all of the tissues in the same way. These facts might be taken into consideration in planning the use of combinations of agents. It would be expected that S-phase-specific agents would be most toxic when they were administered at a time when stimulated DNA synthesis (as indicated by overshoots in the curves) was occurring. Non-phase-specific agents, such as alkylating agents, might be less toxic at those times, since these agents are somewhat less toxic to cells during S phase than during other phases of the cell cycle (23). On the other hand, there is evidence (24) that some alkylating agents, such as cyclophosphamide and BCNU, are more toxic to cycling cells than to noncycling cells, and therefore these agents would be most toxic to tissues when the growth fraction is large. Three experiments were performed to test these expectations.

Influence of Scheduling of Two-Drug Combinations Upon Lethality and [C^3H_3]Thymidine Incorporation

Adriamycin + 5-FU.—Normal mice (28 per group) were given a single ip LD10 dose of adriamycin and a single ip LD10 dose of 5-FU either simultaneously with adriamycin or at 24, 48, 72, or 96 hours after adriamycin. The death patterns and the numbers of 45-day survivors are shown in figure 2. Simultaneous administration of adriamycin and 5-FU resulted in fewer animal deaths than the administration of 5-FU at 24, 48, 72, or 96 hours after adriamycin. In another experiment, mice bearing transplanted C3H-16/C breast tumors were given simultaneous doses of adriamycin and 5-FU or adriamycin followed by 5-FU at 72 hours, and the rates of incorporation of [C^3H_3]thymidine into the DNA of specified tissues and tumors were determined at various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12.26
16.25
15.06
DIFFERENCE FROM MEAN / STANDARD DEVIATION
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+9
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24
72
168
240
336
408
456
HOURS AFTER TREATMENT
MARROW
B
DIFFERENCE FROM MEAN / STANDARD DEVIATION
+10
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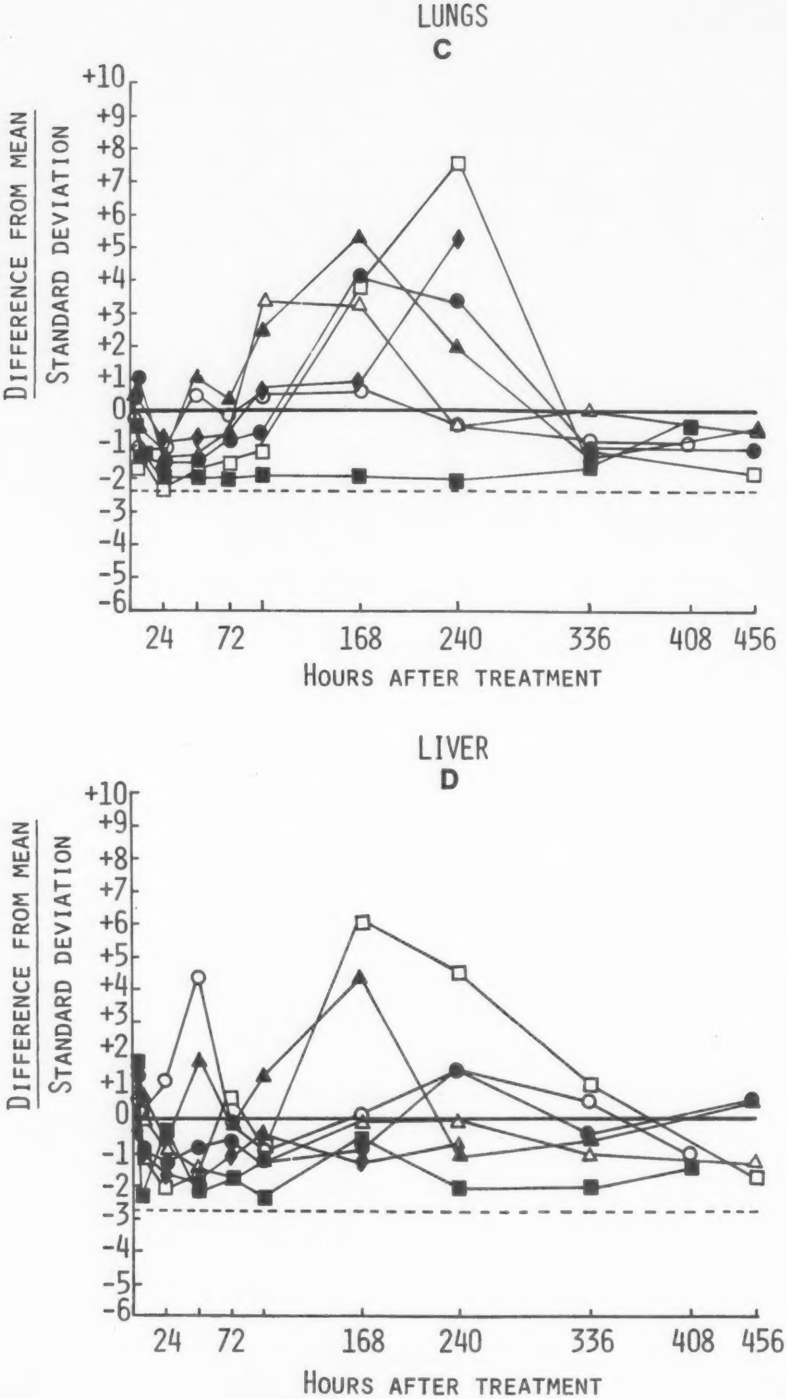
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DIFFERENCE FROM MEAN
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HOURS AFTER TREATMENT
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DIFFERENCE FROM MEAN
STANDARD DEVIATION
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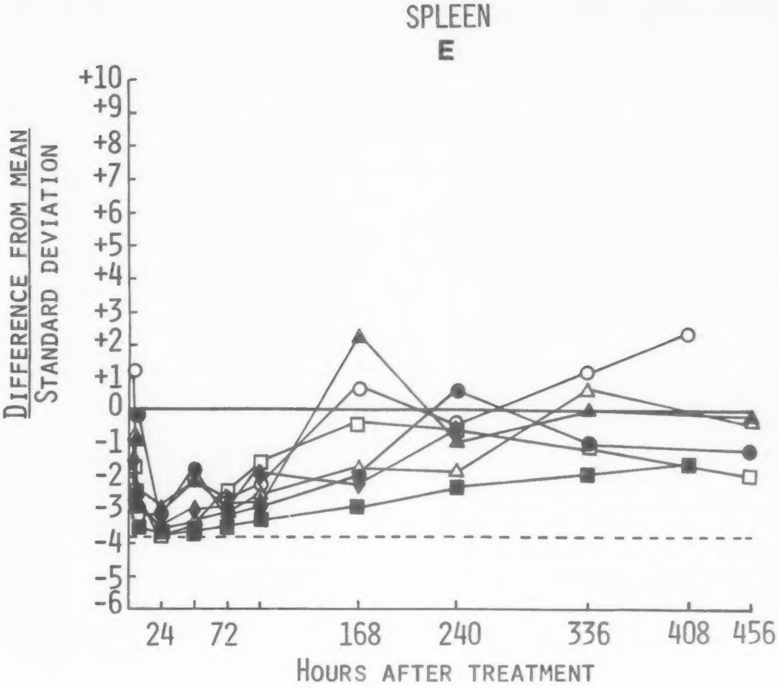
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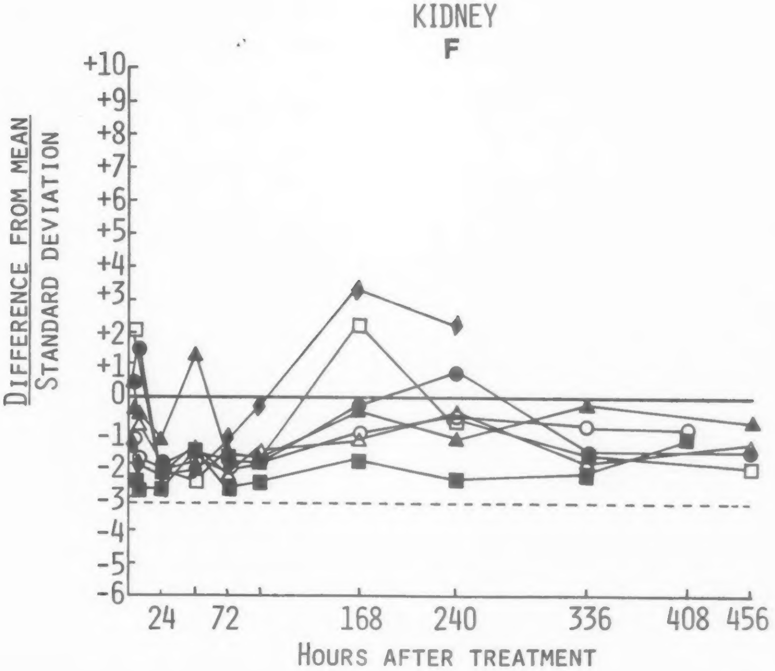
KIDNEY
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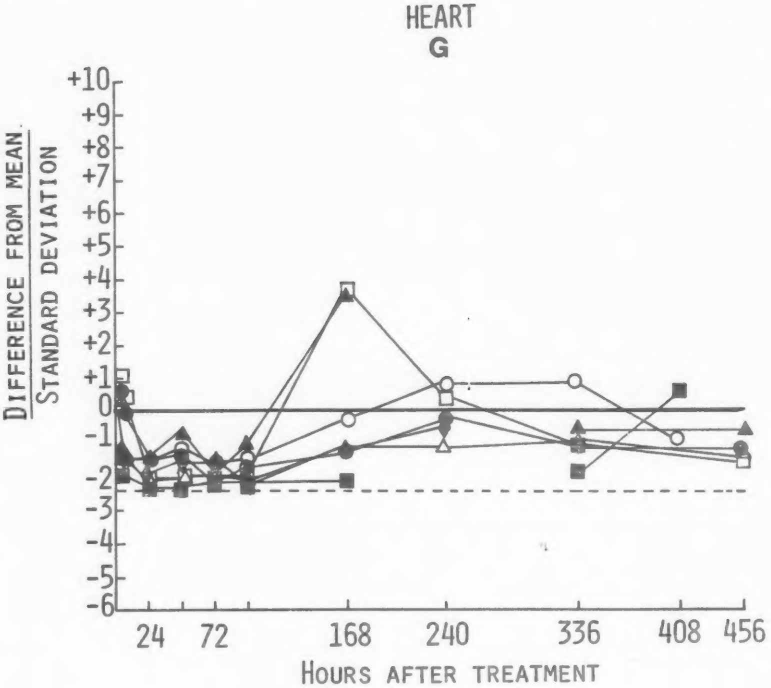

FIGURE 1.—Effects of single ip doses (LD10s) of the various agents upon the incorporation of $[C^3H_3]$thymidine into the DNA of the indicated tissues (A–G), when the thymidine was injected ip at the indicated time after treatment and the animals were killed 2 hrs later. The incorporation was essentially 0 at the values of the dashed lines. ■ = CCNU; ◆ = chlorozotocin; △ = melphalan; ● = adriamycin; ▲ = methotrexate; □ = 5-FU; ○ = vincristine.

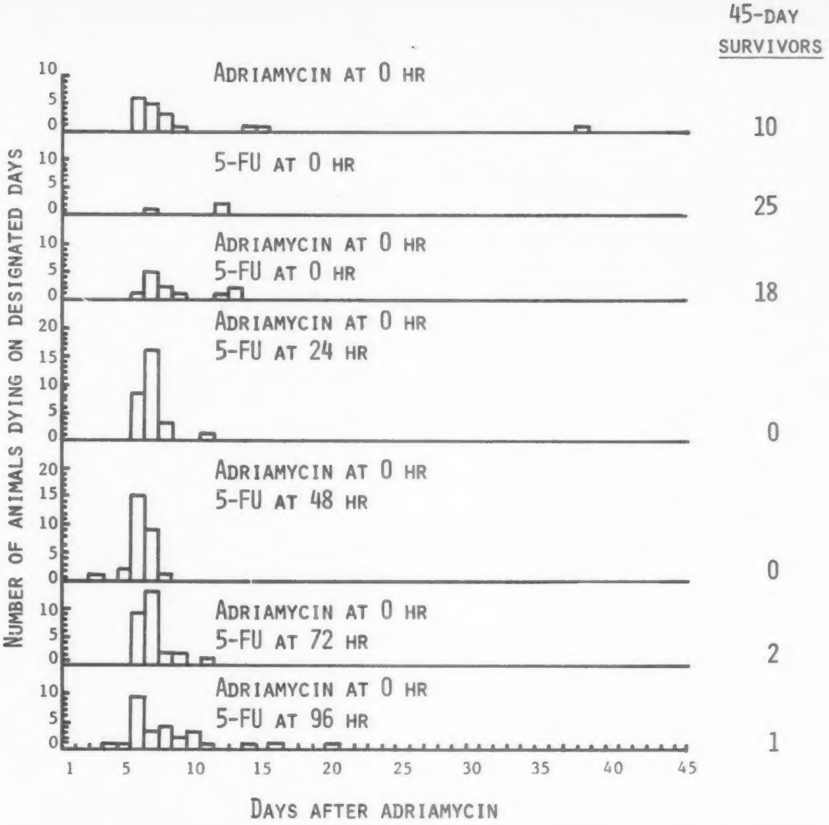

FIGURE 2.—Death patterns for groups of 28 mice that received single ip LD10 doses of adriamycin and 5-FU at the indicated times.

times after administration of the agents. With both dosage schedules the tumors in the surviving mice regressed in size and became impalpable. As shown in figure 3, after the simultaneous administration of adriamycin and 5-FU the pattern of incorporation of [C^3H_3]thymidine by the tissues at the various times was similar to that caused by 5-FU alone, ie, early inhibition of all tissues followed by extensive stimulation of intestinal mucosa at 72 hours, somewhat less stimulation of liver at 168 hours, and even less stimulation of the other tissues at 260 hours. A noticeable difference, however, was the greater inhibition of marrow and the delayed and much smaller "overshoot" by marrow. When adriamycin was given 72 hours before 5-FU, the pattern of incorporation of [C^3H_3]thymidine during the 72-hour period was similar to that observed previously for adriamycin, with early inhibition of incorporation in all tissues and with much stimulation of incorporation in the intestinal mucosa and marrow at 72 hours. Thus, the 5-FU was given at a time when there was extensive stimulation in the mucosa and marrow, significant inhibition in the spleen, lungs, kidneys, and heart, and an approximately normal incorporation into the liver. As would be expected for tissues actively synthesizing DNA at the time of exposure to 5-FU, 24 hours after the administration of 5-FU there was much inhibition in mucosa and marrow. By 48 hours after 5-FU, the incorporation by the mucosa was approximately normal, and by 96 hours it was greatly stimulated. By contrast, at 48 hours after the 5-FU, the incorporation by marrow was undetectable, and it was still undetectable at 96 hours, at which time the experiment was terminated because unexpected excessive deaths (probably resulting from drug toxicity) depleted the supply of animals. The data of figures 2 and 3 show that the deaths of the animals on both schedules occurred when the DNA synthesis by the marrow was minimal and the DNA synthesis by the intes-

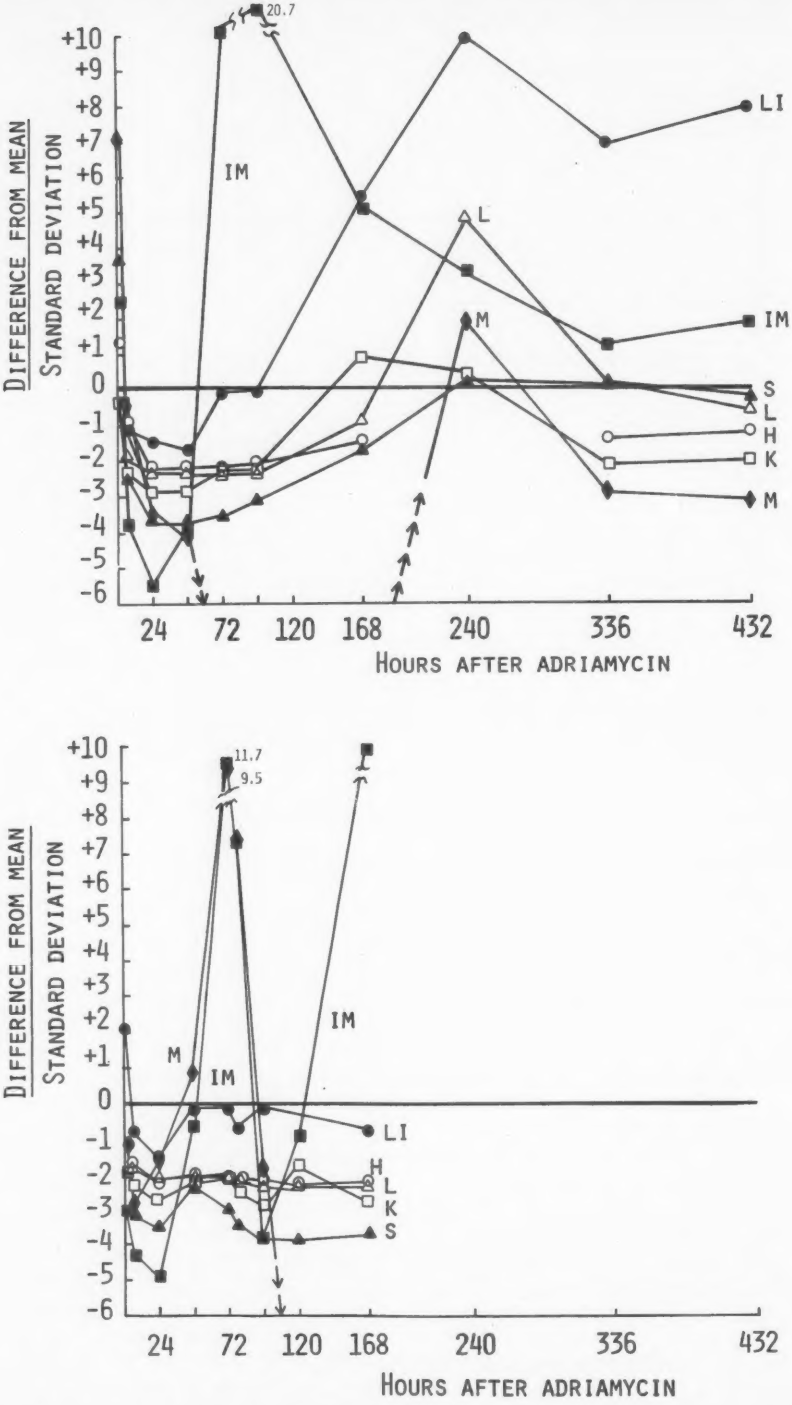

FIGURE 3.—Effects of combinations of single ip LD10 doses of adriamycin and 5-FU upon the incorporation of [C^3H_3]thymidine into the DNA of various tissues. The data for the upper graph were obtained when the agents were administered simultaneously at 0 hr. The data for the lower graph were obtained when adriamycin was given at 0 hr and 5-FU was given at 72 hrs. M = marrow; IM = intestinal mucosa; LI = liver; L = lungs; K = kidneys; H = heart; S = spleen.

tinal mucosa was stimulated. The results of these experiments are consistent with those of other investigators (5,10,11) who administered two successive doses of 5-FU and observed that animal lethality was greatest when the second dose was given at a time of stimulated synthesis of DNA by marrow and intestinal mucosa following the first dose. Figure 4 shows the effects of the treatments upon the incorporation of [C^{3}H$_{3}$]thymidine by the tumors in the above experiment. The large increase of incorporation at 0 hour by the tumors of animals that received the two agents simultaneously is probably due to the decreased pool of endogenously synthesized nonradioactive thymidine phosphate. During the period from 24 to 336 hours the tumors regressed to very small sizes and very little incorporation occurred; the large amount of incorporation that occurred at 432 hours accompanied the regrowth of the tumors. In agreement with experimental results obtained previously in this laboratory and elsewhere with other tumors, the rate of incorporation of [C^{3}H$_{3}$]thymidine by the tumor remained depressed at times when the other tissues had returned to or had exceeded normal rates. When adriamycin was administered prior to the 5-FU, some recovery by the tumors of the ability to incorporate [C^{3}H$_{3}$]thymidine occurred by 48 hours, but after administration of 5-FU at 72 hours the tumors regressed and incorporation of [C^{3}H$_{3}$]-thymidine was not detected.

Adriamycin + CCNU.—Another toxicity experiment showed (fig 5) that simultaneous administration of adriamycin and CCNU resulted in earlier deaths than the administration of CCNU ≥ 24 hours after the adriamycin. Administration of CCNU at 48 hours after adriamycin caused more and earlier deaths than administration at 24, 72, or 96 hours after adriamycin. Therefore, a [C^{3}H$_{3}$]thymidine incorporation experiment in which CCNU was administered to tumor-bearing mice either 24 or 48 hours after administration of adriamycin was performed. It is evident (fig 6) that the two schedules resulted in different patterns of incorporation of [C^{3}H$_{3}$]thymidine into the DNA of mucosa and marrow. Comparison of the curves of figure 6 with the curves for the LD10 dose of adriamycin in figure 1 shows that when CCNU was administered at 24 hours after adriamycin, a time when the incorporation by marrow was normal or slightly depressed and the incorporation by mucosa was greatly depressed, the "overshoots" for these tissues were delayed, there was a secondary "overshoot" by intestinal mucosa, and there was no great inhibition of marrow. With the exception of the liver, there was little recovery by the other tissues during the period of the experiment. If CCNU was administered at 48 hours after adriamycin, a time when the incorporation by marrow was somewhat stimulated and the incorporation by mucosa was recovering, the incorporation by marrow was depressed to

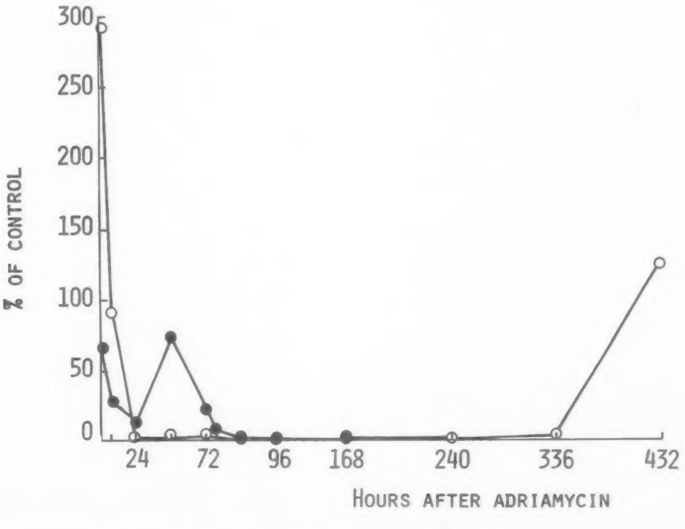

FIGURE 4.—Effects of combinations of single ip LD10 doses of adriamycin and 5-FU upon the incorporation of [C^{3}H$_{3}$]thymidine into the DNA of C3H-16/C tumors. ● = adriamycin given at 0 hr and 5-FU given at 72 hrs; ○ = adriamycin and 5-FU given simultaneously at 0 hr.

a nondetectable level for a number of days, and recovery by all of the other tissues, including mucosa, was delayed but was somewhat greater than when the interval between agents was 24 hours. With both schedules the incorporation of [C^3H_3]thymidine by the tumor was greatly reduced (fig 7), and the tumors had completely regressed. The data of figures 1, 5, and 6 show that greater lethality occurred if CCNU was given when the DNA synthesis of the marrow and the intestinal mucosa was normal (simultaneous with adriamycin) or stimulated (48–72 hours after adriamycin) rather than when it was low (24 hours after adriamycin). These results are consistent with the results reported by Young et al (14) and Brereton et al (16) who administered a single dose of methyl-CCNU or two one-half doses of methyl-CCNU spaced at various time intervals; greater lethality occurred with the single dose or with administration of the second one-half dose at a time of normal or stimulated DNA synthesis by the marrow and intestinal mucosa.

Methotrexate + 5-FU.—Mice were randomly allocated to seven groups of 15 mice each. The mice of each group received a single dose of methotrexate (100 mg/kg) and a single dose of 5-FU (180 mg/kg) in a specified sequence and at specified times. One group received the two agents simultaneously (the injection of the second agent immediately followed the injection of the first). Other groups received injections of the two agents spaced at 24-, 48-, or 120-hour intervals. The groups were checked daily for dead animals and the numbers of dead animals were recorded. Figure 8 shows when deaths occurred in the various groups of animals. The greatest lethality, resulting in the death of all of the mice in the group, resulted when methotrexate was given 120 hours after 5-FU. Administration of 5-FU 120 hours after methotrexate resulted in the next greatest lethality. Little or no lethality occurred when the two agents were given simultaneously or were spaced at 24 or 48 hours regardless of which agent was given first. On the basis of the curves for these agents in figure 1 and information published by others, one can suggest reasons for the greater lethality of the combination of the two agents when they are given 120 hours apart.

It has been reported that cells are most sensitive to methotrexate when they are in S, early-G_1, or G_1/S phase (23), and that they are sensitive to 5-FU throughout the cycle (19) but that cycling cells are more sensitive than non-cycling cells to 5-FU (24).

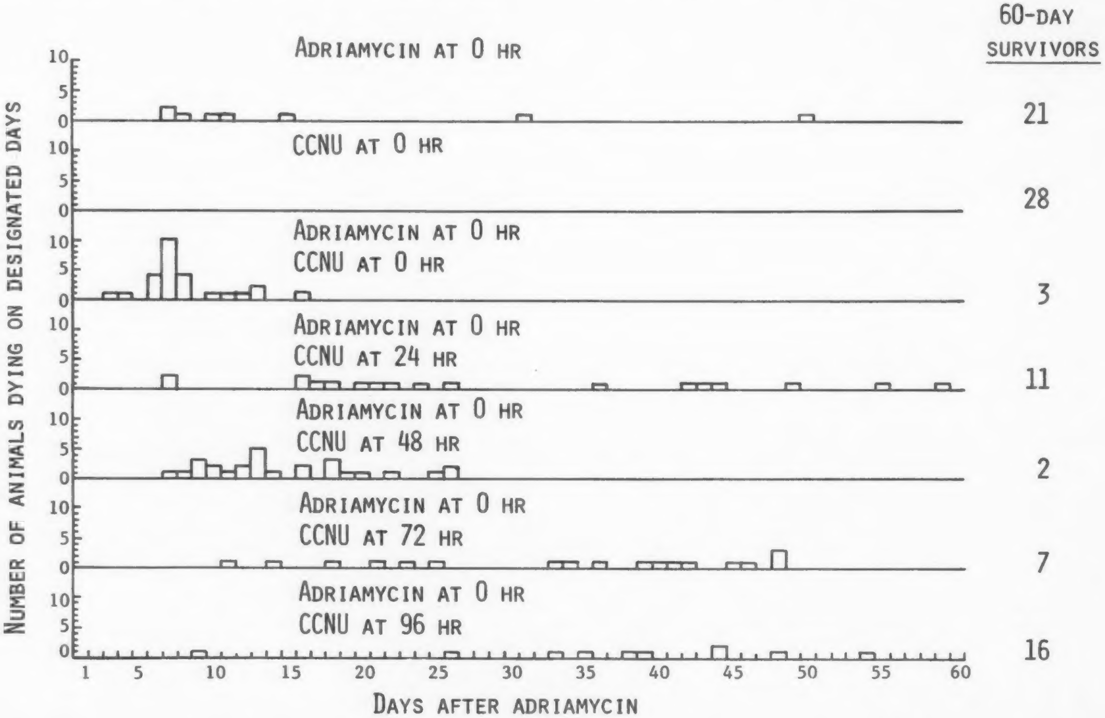

FIGURE 5.—Death patterns for groups of 28 mice that received single ip LD10 doses of adriamycin and CCNU at the indicated times.

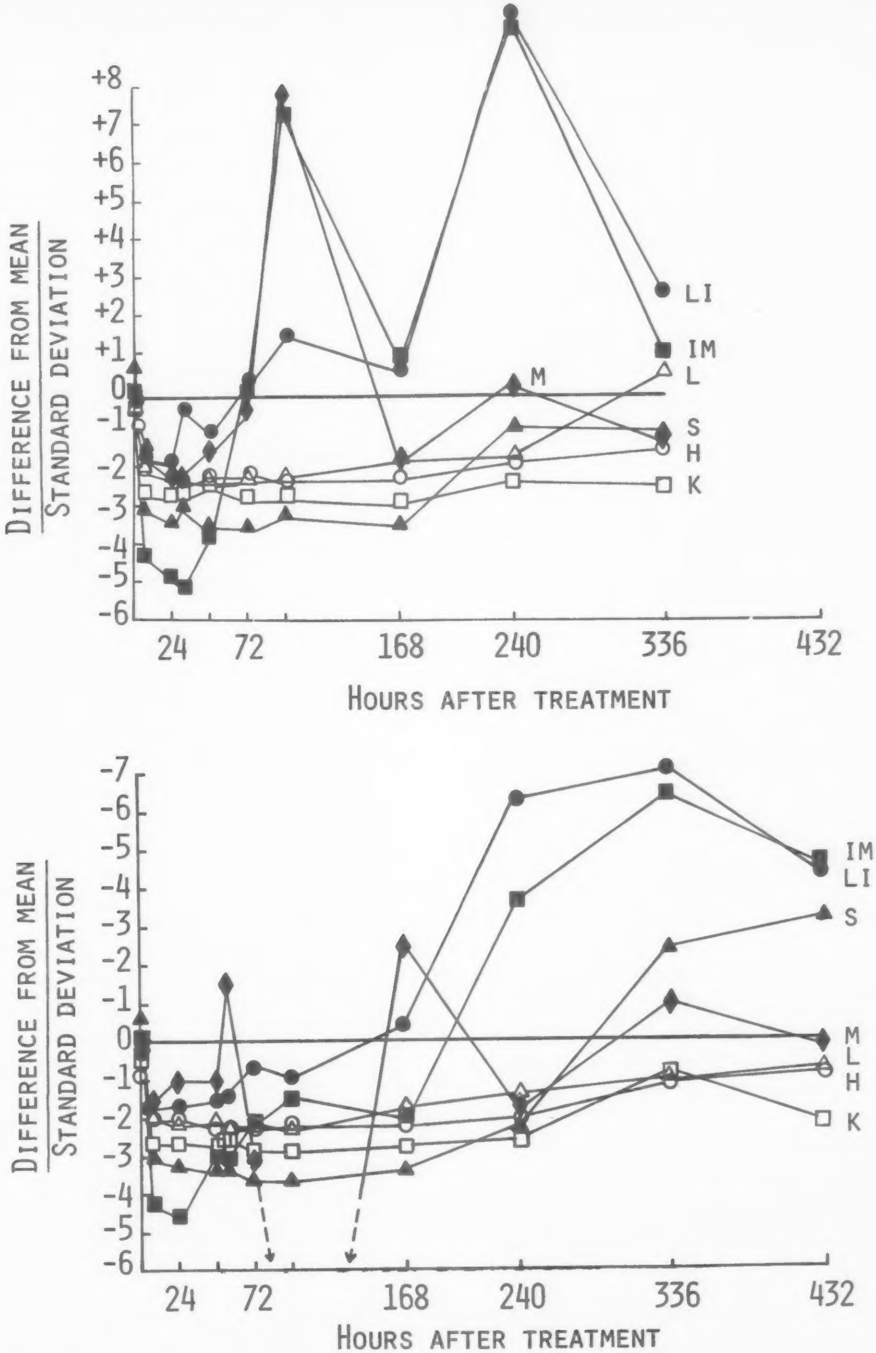

FIGURE 6.—Effects of combinations of single ip LD10 doses of adriamycin and CCNU upon the incorporation of [C^{3}H$_3$]thymidine into the DNA of various tissues. The data for the upper graph were obtained when the adriamycin was given at 0 hr and the CCNU was given at 24 hrs. The data for the lower graph were obtained when adriamycin was given at 0 hr and CCNU was given at 48 hrs.

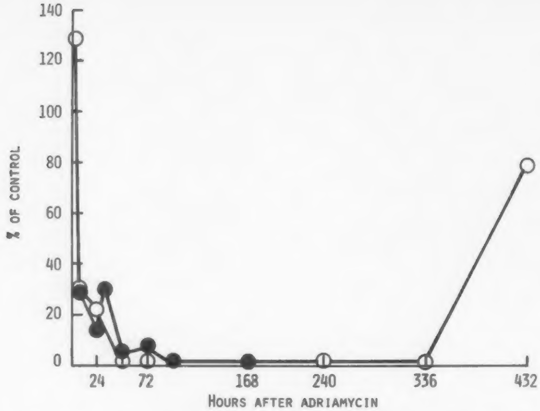

FIGURE 7.—Effects of combinations of single ip LD10 doses of adriamycin and CCNU upon the incorporation of [C^3H_3]thymidine into the DNA of C3H-16/C tumors. ● = adriamycin given at 0 hr and CCNU given at 24 hrs; ○ = adriamycin given at 0 hr and CCNU given at 48 hrs.

At the time of initial treatment of the mice it is likely that the growth fractions of many of the tissues are rather low and therefore the toxicity of methotrexate and 5-FU would be relatively low for those tissues, and hence for the whole animal. At 24 hours after either agent alone, the decreased incorporation of thymidine indicates that little DNA is being synthesized and that sensitivity to the second agent might be low at that time. By 48 hours after methotrexate, the stimulated incorporation of thymidine probably indicates that more cells are cycling than would be cycling in untreated tissues, and this stimulation continues for up to 168 hours. During this interval there might be increased sensitivity to 5-FU. Between 24 and 96 hours after a single dose of 5-FU, there was a low rate of synthesis of DNA by all of the tissues except intestinal mucosa, and therefore the sensitivity to methotrexate might be low. However, by 120 hours, there was stimulated synthesis of DNA and therefore the tissues might be more sensitive to methotrexate.

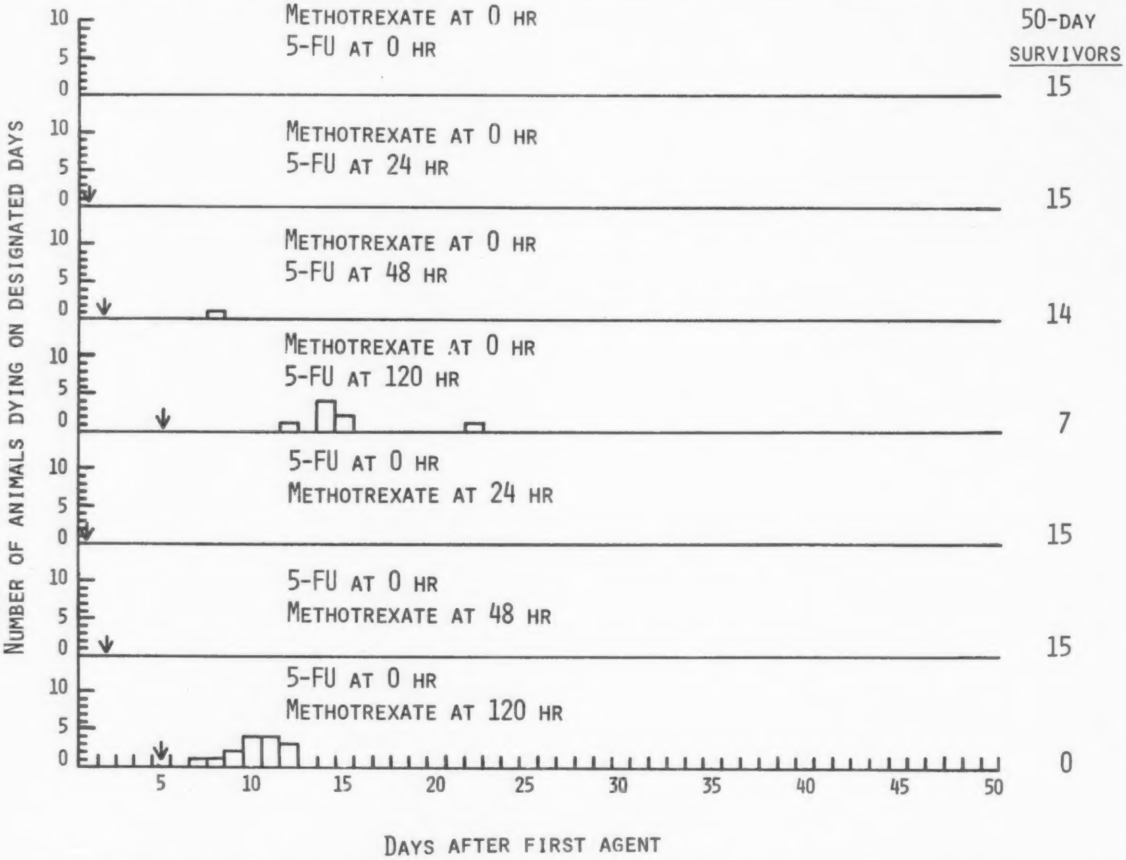

FIGURE 8.—Death patterns for groups of 15 mice that received single ip LD10 doses of methotrexate and 5-FU at the indicated times. The arrows = the time at which the 2nd agent was given.

CONCLUSIONS

These results indicate that information on the effects of individual agents upon DNA synthesis of tissues at various times after administration of the agents might be used beneficially in conjunction with other available information about the effects of the agents to plan chemotherapy regimens with low or minimal drug toxicity. Although most of the agents tested in this study caused inhibition of DNA synthesis in all of the tissues examined (intestinal mucosa, marrow, lungs, liver, spleen, kidneys, and heart), in most instances the greatest inhibitions and the greatest "overshoots" occurred in intestinal mucosa and marrow, and the data obtained in the experiments with two-drug combinations indicated that the effects upon these two tissues were the most useful for planning schedules for minimal toxicity to the animal. These results for an experimental system are gratifying, because toxicity to marrow and to intestinal mucosa are frequently limiting factors in clinical chemotherapy.

This study serves to confirm the studies of Young et al and to supplement them by providing data for host tissues other than marrow and intestinal mucosa and by testing two-drug combinations. The results of the two-drug experiments are consistent with the results obtained by Young for multiple doses of a single agent (5), by indicating that if CCNU, 5-FU, or methotrexate is the second agent given, it should be given at a time when the rate of synthesis of DNA is low to minimize toxicity to the host.

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Comparison of Adriamycin-Induced Nucleolar Segregation in Skeletal Muscle, Cardiac Muscle, and Liver Cells[1,2]

Jerome Merski, Yerach Daskal, and Harris Busch[3,*]

SUMMARY

Male Sprague-Dawley rats were treated with either 2.5, 3.5, or 5.0 mg/kg of adriamycin by iv injection. After 1 or 3 hours of treatment, samples of liver, cardiac muscle, and skeletal muscle cells were examined by electron microscopy. The changes in ultrastructure observed in these tissues after the first hour included nucleolar segregation and altered distribution of the perinucleolar chromatin. However, no alterations in the ultrastructure of either the nucleus or cytoplasm were observed in tissues examined 3 hours after a 5-mg/kg dose of adriamycin. The doses at which nucleolar alterations occurred varied between tissues. In skeletal and cardiac muscle cells, marked alterations in nucleolar ultrastructure were observed at doses of 3.5 and 5.0 mg/kg of adriamycin. Liver cell nucleoli, however, exhibited few structural aberrations at these doses. The similarities in response of skeletal and cardiac muscle suggest that ultrastructural analysis of skeletal muscle biopsies may be useful in evaluating adriamycin cardiotoxicity.

[Cancer Treat Rep 62:771–778, 1978]

Adriamycin is an anthracycline antibiotic isolated from cultures of *Streptomyces peucetius* var. *caesius*. Since its introduction, widespread interest has developed in the effectiveness of this drug as a cancer chemotherapeutic agent (1–4) as well as in its characteristic side effect of congestive heart failure (2–7). Clinical studies determined that the frequency of adriamycin-related heart failure increased significantly when the total dose administered exceeded approximately 550 mg/m^2 (4,6). By limiting the total dose of drug below this level, the often fatal cardiotoxic effects could generally be avoided. These restrictions, however, limit the usefulness of the drug, particularly in patients with tumors that are responsive to therapy.

One way to reduce these problems is through the development of a safe, accurate method for predicting on an individual basis whether adriamycin therapy may safely be extended beyond the presently established dose limits. Up to now, methods used to predict the occurrence of cardiomyopathy have included electrocardiography, echocardiography, ratios of pre-ejection period to left ventricular emptying time, serum enzyme levels, and most recently, endocardial biopsies (5–10). Unfortunately, these techniques have not proven altogether adequate (5–7).

Another method of approaching the problems involved in long-term adriamycin therapy is to define the cardiotoxic mechanism of adriamycin at the cellular level. It is hoped that by understanding the mechanism involved in this toxicity, effective counter-measures can be developed. Several possible mechanisms have been suggested based on results derived in small animal models (11–18), but definitive evidence for any one of these is still lacking.

In the present study, evidence is presented to demonstrate similarities in the response of skeletal and cardiac muscle cell nucleoli to adriamycin treatment. In addition, differences in response to low doses of adriamycin were observed between skeletal and cardiac muscle cell and liver cell nucleoli.

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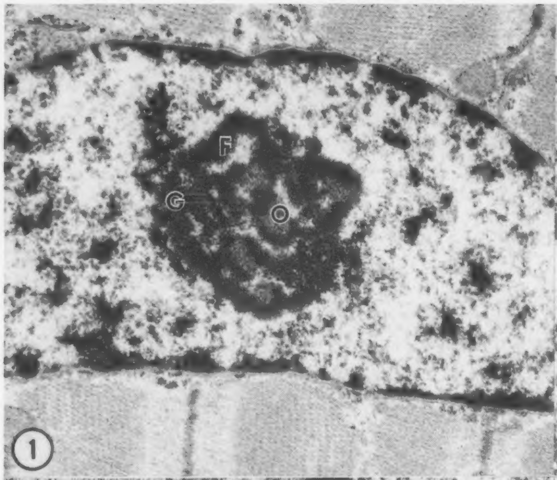

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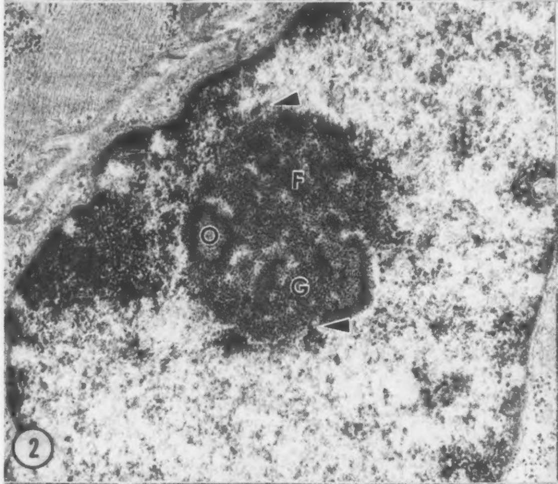

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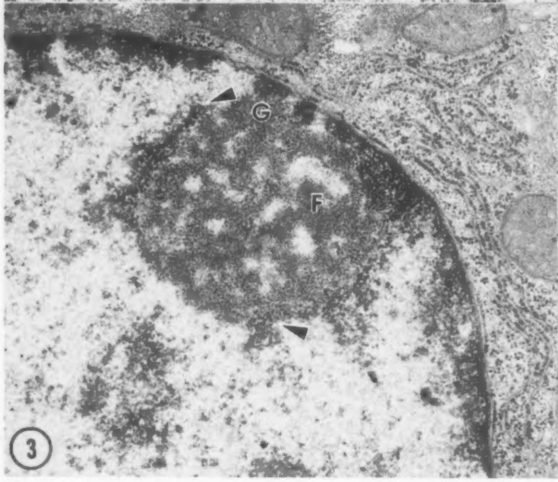

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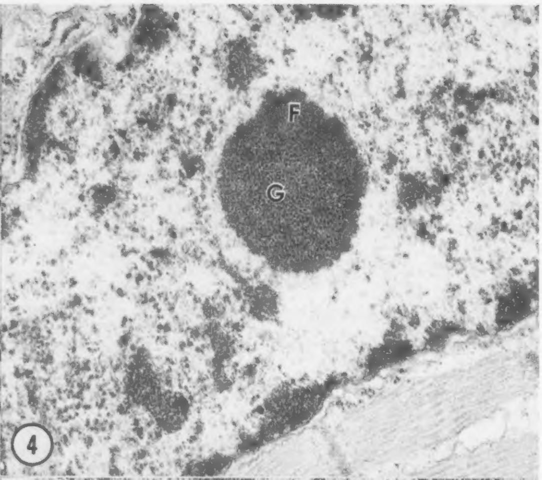

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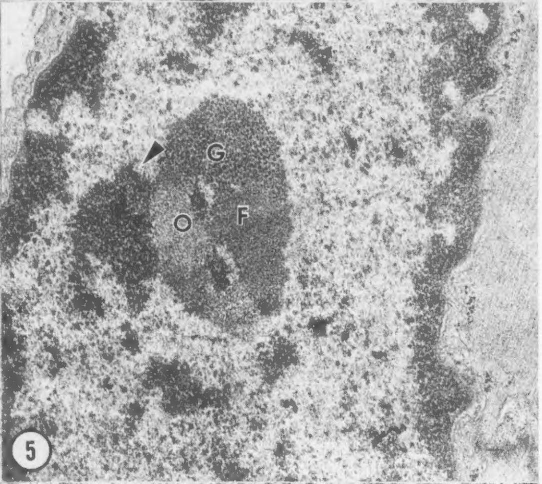

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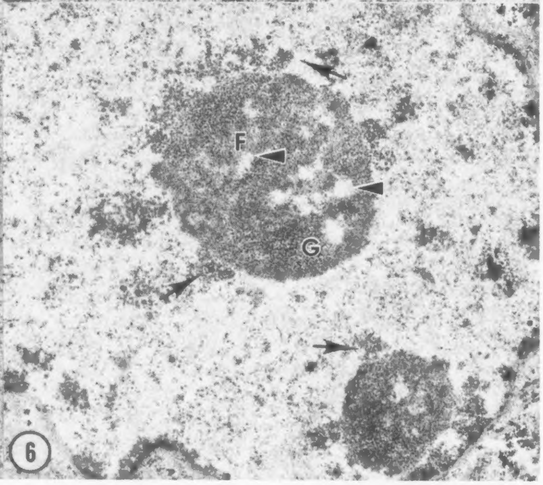

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FIGURE 1.—Nucleus of a control ventricular muscle cell. The nucleolus is composed of randomly distributed granular (G), fibrillar (F), and nucleolar organizer (O) components in a characteristic trabecular structure (× 20,000).

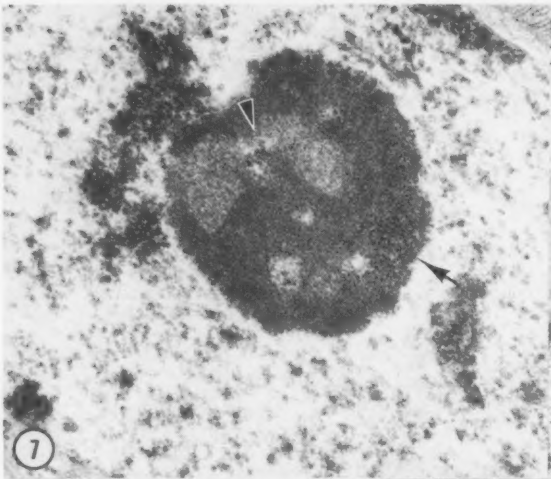
FIGURE 2.—Nucleus of skeletal muscle cell that was not exposed to adriamycin treatment. The granular (G), fibrillar (F), and nucleolar organizer (O) components are distributed randomly in the nucleolus. The perinucleolar chromatin (pointer) is loosely attached to the nucleolar surface (uranyl acetate-lead citrate stain, × 23,750).

FIGURE 3.—Nucleus of liver cell that was not exposed to adriamycin treatment. The nucleolar organizer (O), granular (G), and fibrillar (F) components appear in a typical random arrangement with perinucleolar chromatin attached to the nucleolar periphery (pointer) (uranyl acetate-lead citrate stain, × 23,750).

FIGURE 4.—Nucleolus (highly segregated) of ventricular muscle cell 1 hr after treatment with 5 mg/kg of adriamycin. The granular (G) and fibrillar (F) components appear in clearly defined areas at opposite ends of the nucleolus. The perinucleolar chromatin is absent from the nucleolar surface (uranyl acetate-lead citrate stain, × 27,500).

FIGURE 5.—Nucleus of skeletal muscle cell 1 hr after treatment with 5 mg/kg of adriamycin. The granular (G), fibrillar (F), and nucleolar organizer (O) components occupy discrete areas and the perinucleolar chromatin (pointer) is highly condensed (uranyl acetate-lead citrate stain, × 31,250).

FIGURE 6.—Nucleoli of this liver cell appears compact 1 hr after treatment with 5 mg/kg of adriamycin, but remnants of the normal trabecular structure (pointers) are still visible. In addition, the granular (G), fibrillar (F), and nucleolar organizer (O) components have not segregated. The perinucleolar chromatin condensed to form several small chromocenters (arrows) close to the nucleolus (uranyl acetate-lead citrate stain, × 31,250).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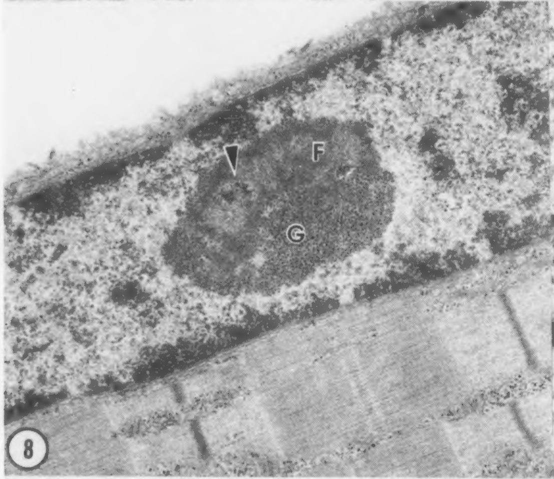
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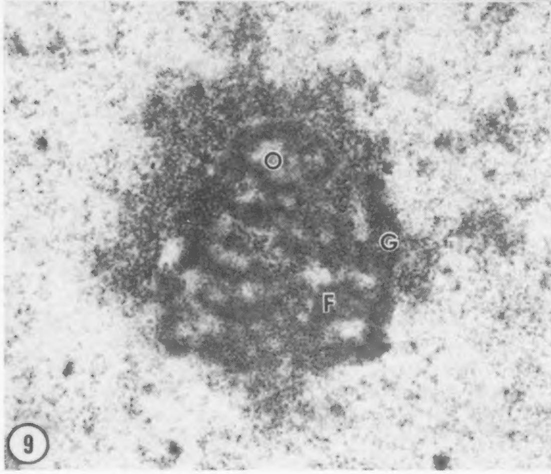
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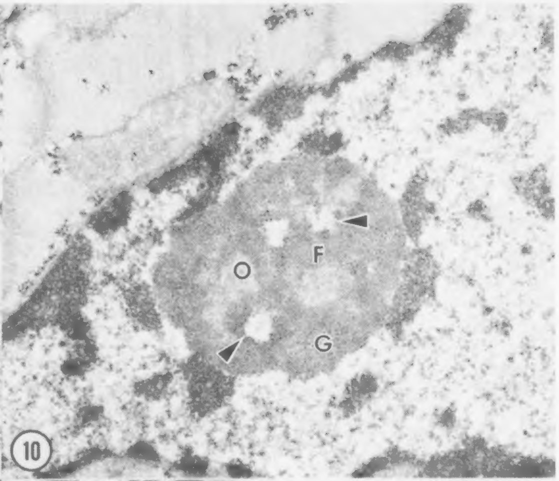
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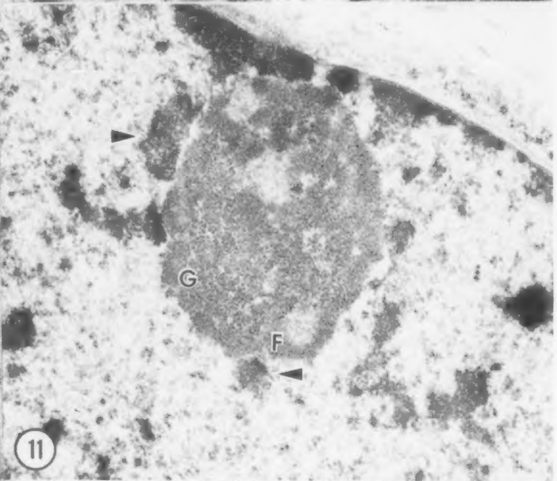
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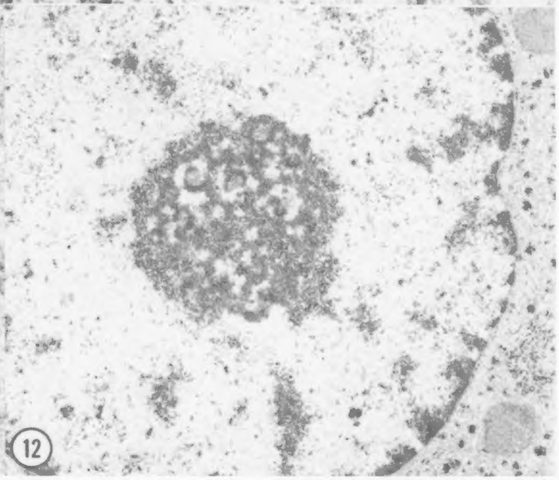
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FIGURE 7.—Nucleolus of ventricular muscle cell 1 hr after treatment with 3.5 mg/kg of adriamycin. Nucleolar organizer components have begun to fuse (pointer) and move toward the periphery of the nucleolus. Only a small amount of granular component is present (arrow) (uranyl acetate-lead citrate stain, × 42,500).

FIGURE 8.—Nucleus of skeletal muscle cell 1 hr after treatment with 3.5 mg/kg of adriamycin. The granular (G) and fibrillar (F) components of the nucleolus have begun to segregate. Nucleolar organizer regions have fused (pointer) and moved to the outer edge of the nucleolus (uranyl acetate-lead citrate stain, × 22,500).

FIGURE 9.—Nucleolus of liver cell 1 hr after treatment with 3.5 mg/kg of adriamycin. The granular (G), fibrillar (F), and nucleolar organizer (O) components are distributed in a random pattern typically found in an untreated cell (uranyl acetate-lead citrate stain, × 25,000).

FIGURE 10.—Nucleolus of ventricular muscle cell 1 hr after treatment with 2.5 mg/kg of adriamycin appears compact with only a few indications of trabecular structure remaining (pointers). The granular (G), fibrillar (F), and nucleolar organizer (O) components are mixed in a relatively random pattern throughout the nucleolus (uranyl acetate-lead citrate stain, × 23,100).

FIGURE 11.—A random distribution of the granular (G), fibrillar (F), and nucleolar organizer (O) components is evident in this nucleolus of a skeletal muscle cell 1 hr after treatment with 2.5 mg/kg of adriamycin. Some clumping of the perinucleolar chromatin (pointers) is still present, however, (uranyl acetate-lead citrate stain, × 23,750).

FIGURE 12.—Alterations in the structure of the nucleus or nucleolus are absent in the liver cell following 1 hr of treatment with 2.5 mg/kg of adriamycin (uranyl acetate-lead citrate stain, × 13,000).

MATERIALS AND METHODS

Adriamycin (Adria Laboratories, Inc, Wilmington, Del) was purchased in sealed vials containing 10 mg of drug and 50 mg of lactose. The contents of each vial were reconstituted with normal saline to give a final concentration of 1 mg/ml. Male Sprague-Dawley rats (180–250 g) were administered the reconstituted drug by iv injection. Each group of animals (three per group) received either 2.5, 3.5, or 5.0 mg/kg of adriamycin. Control animals received an equivalent volume of saline.

One hour after treatment, one group of animals from each dose group was killed by decapitation. One group of animals that had received 5.0 mg/kg of adriamycin was killed 3 hours after treatment. Portions of the left ventricular wall, liver, and skeletal muscle were removed from each rat. After excision, muscle samples were pinned on dental wax covered with fixative. Primary fixation was carried out in a solution of 4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.4). All samples were postfixed in a 2% solution of osmium tetroxide. Dehydration in a graded series of alcohol solutions was followed by embedding in epon-araldite. Silver-gray sections of skeletal muscle, cardiac muscle, and liver from each animal in the respective dose groups were cut on a Sorvall MT-2 ultramicrotome with a diamond knife. Sections were examined and photographed on a Phillips EM 200 electron microscope.

RESULTS

One hour after treatment with 5 mg/kg of adriamycin the cytoplasmic components of the liver, skeletal muscle, and cardiac muscle cells appeared essentially unchanged. However, changes in the structure of nuclei and particularly in the nucleoli of these tissues were present at this time. The most pronounced effect was observed in the nucleoli of the cardiac and skeletal muscle cells.

Prior to treatment, the skeletal and cardiac muscle cell nucleoli (figs 1 and 2) appeared characteristic of nucleoli actively synthesizing RNA. They had a trabecular appearance with nucleolar organizer, granular, and fibrillar components randomly distributed. Following the 5-mg/kg dose of adriamycin, nucleoli of cardiac and skeletal cells were compact and clearly segregated (figs 4 and 5). In these instances, the nucleolar organizer elements anastomosed and were located at the nucleolar periphery. The granular and fibrillar components segregated into discrete regions and in some instances separated from the main portion of the nucleolus. Changes in the nuclear chromatin were also observed. These included the formation of chromocenters and migration of the perinuclear chromatin away from the nucleolus. Three hours after treatment, the ultrastructure of both the cytoplasmic and nuclear components was comparable to that of control cells.

The effect of the 5-mg/kg dose of adriamycin on liver cell nucleoli was not as marked as in the cardiac and skeletal muscle cells described above. Figure 3 represents a characteristic, untreated liver cell nucleolus. After treatment, the liver cell nucleoli were compact, but remnants of the characteristic trabecular structure were still present and segregation was not observed (fig 6). In addition, the nucleolar organizer component did not fuse or move toward the nucleolar periphery. Changes in the chromatin were also less pronounced as the perinucleolar chromatin remained closely associated with the nucleolar surface. Even these slight alterations, however, were not observed in samples from animals killed 3 hours after treatment.

One hour after the 3.5-mg/kg dose of adriamycin the difference in effect with respect to nucleolar alterations between the cardiac and skeletal muscle and liver cell nucleoli was most obvious (figs 7–9). At this dose, the skeletal and cardiac muscle cell nucleoli show distinct signs of segregation (figs 7 and 8), although less pronounced than those observed after the 5-mg/kg treatment. The liver cell nucleoli of the treated rats (fig 9), however, were indistinguishable from nucleoli of control animals (fig 3).

Only minimal changes were observed in the nucleoli of skeletal and cardiac muscle cells following treatment with 2.5 mg/kg of adriamycin (figs 10 and 11). These changes included a partial collapse of the trabecular structure and some segregation of the granular and fibrillar components. Alterations of liver cell nucleoli were not observed following treatment with 2.5 mg/kg of adriamycin (fig 12).

The observations described above are representative of approximately 90% of the cell population in the tissues examined in each dose group; the only exception was a response of approximately 60% in the skeletal muscle cell nucleoli in one animal in the 3.5-mg/kg dose group.

DISCUSSION

Nucleolar segregation has been demonstrated in cardiac muscle, skeletal muscle, liver, and Novikoff hepatoma ascites cells following exposure to adriamycin either in vivo or in vitro (15,16,19,20). Despite exposure to very high doses (16), these effects were always transient in liver cells. Depending on the dose used and the time of exposure, however, cardiac muscle cell nucleoli did not always recover within the experimental period (16). In addition, it has been suggested that nucleolar damage may be involved in the genesis of cytoplasmic lesions characteristic of chronic adriamycin toxicity, despite the reappearance of normal nucleolar ultrastructure in these cells (15).

In the present study, a close correlation was found between the response of skeletal and cardiac muscle cell nucleoli and treatment with low doses of adriamycin. Although of limited duration, such alterations indicate that inhibition of RNA synthesis and possibly other toxic actions of adriamycin may occur in a similar fashion in both cardiac and skeletal muscle cells.

Recently, electron microscopic data obtained from endocardial biopsies of patients receiving adriamycin therapy were found to show progressive myocardial damage which was not detected by conventional measures of cardiac function (8). If similar structural changes do occur in both cardiac and skeletal muscle cells, it may be possible to develop a distinctive set of structural markers useful in predicting the presence of adriamycin-induced cardiac damage. Such a technique would decrease the risk involved in obtaining this information from endocardial samples. Work is presently being carried out to further investigate this possibility.

As noted in the results, a 3.5-mg/kg dose of adriamycin caused marked alterations in the ultrastructure of cardiac and skeletal muscle cell nucleoli. A dose of 5.0 mg/kg, however, was necessary to produce similar changes in liver cell nucleoli. In a previous study (16), another example of selective action by adriamycin on nucleoli of cardiac muscle cells was reported. The basis for this apparent selectivity by adriamycin in either case is not known. However, the reproducibility of this effect and its apparent specificity for anthracycline antibiotics (unpublished data) suggest that it may be of value in testing anthracycline derivatives for cardiotoxic properties.

Suggestions for the mechanism of adriamycin cardiotoxicity include decreased synthesis of RNA (13,15,16), cell damage caused by adriamycin-associated free radical formation (21), and inhibition of mitochondrial enzymes (22). Since supportive biochemical evidence exists for all three of these effects (1,21,22), it seems possible that all three may be involved in the genesis of this toxicity. If so, the effect of inhibition of RNA synthesis could be amplified and would be of particular importance because of the increased demand of the myocardial cells for mitochondrial, membrane, and other proteins damaged by adriamycin or its metabolites.

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Comparative Antitumor Activity of Actinomycin Analogs in Mice Bearing Ridgway Osteogenic Sarcoma or P388 Leukemia [1,2]

William C. Rose,* Mary W. Trader, W. Russell Laster, Jr, and Frank M. Schabel, Jr [3]

SUMMARY

Actinomycin (Act) analogs, differing in the chemical substitution(s) made at various positions in either their pentapeptide chain(s) or chromophore ring, were evaluated for their antitumor activity in mice bearing either Ridgway osteogenic sarcoma (ROS) or P388 leukemia. Of the analogs tested against advanced (2–3-g) ROS tumors, azetomicin I and Act III caused therapeutic responses which, although variable, were nevertheless indicative of antitumor activities greater than was found using Act D. Several other analogs, Act C_2, 2-*N*-(γ-hydroxypropyl)-Act D, Act $X_0\delta$, and azetomicin II, displayed antitumor activity in ROS-bearing mice which varied, in different experiments, from comparable to superior to that achieved using Act D. Additionally, Act $Pip_1\beta$ and 3′-(4-*cis*Cl-Pro)-Act were comparable to, and Act-2-hydroxy-C_3 inferior to, Act D in activity against ROS. Both azetomicin I and II were as effective as Act D in mice bearing P388 leukemia. Moreover, a subline of P388 that is resistant to Act D was cross-resistant to both azetomicin I and II.

[Cancer Treat Rep 62:779–789, 1978]

Actinomycin D (Act D) was first isolated from *Streptomyces* in 1940 by Waksman and Woodruff (1). It is generally agreed that its primary molecular mechanism of action is expressed through binding to DNA and subsequent inhibition of DNA-dependent RNA synthesis (2).

Act D has been shown to be a clinically useful antitumor agent. It is capable of curing some women with gestational choriocarcinoma (3), as well as inducing complete remissions in some patients with relapsing or persistent choriocarcinoma following initial therapy with methotrexate (4). When used alone or in combination with other therapies, Act D is also useful in the treatment of Wilms' tumor, embryonal rhabdomyosarcoma, metastatic testicular cancer, Ewing's and Kaposi's sarcomas, and malignant melanoma (3). However, a variety of abnormalities of the gastrointestinal, dermal, and hematopoietic systems have been reported in patients receiving Act D (3).

The study of Act analogs was undertaken in an attempt to find those with greater antitumor activity than Act D. Changes in biologic activity of Act analogs reportedly have been obtained by substitutions made in either the cyclic pentapeptide side chains or the chromophore (5,6). We therefore selected for evaluation representatives of several chemical substitution classes of Act analogs, some with known preliminary indications of antitumor activity (Johnson R, NCI, personal communication). In performing our comparisons, we chose two murine tumors known for their marked and reproducible susceptibility to Act D, Ridgway osteogenic sarcoma (ROS) (7–10) and P388 leukemia (9,10). In addition, we evaluated certain of the more active analogs for their antileukemic activity against a subline of P388 leukemia resistant to Act D.

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MATERIALS AND METHODS

Mice

AKR, AKD2F$_1$ [(AKR × DBA/2)F$_1$], DBA/2, and BDF$_1$ [(C57BL/6 × DBA/2)F$_1$] mice, 6–10 weeks old, were used in these experiments. Wayne Lab Blox (Allied Mills, Chicago, Ill) and water were provided ad libitum.

Tumors

ROS was maintained in serial passage at 3-week intervals in AKR mice by sc trocar implant of 150-mg fragments. All chemotherapeutic trials were done using AKD2F$_1$ mice, which we have found to be more suited for aggressive chemotherapy than the AKR mouse. ROS is essentially uniformly fatal in either host, having a $< 1\%$ spontaneous regression rate. The median lifespan of ROS-implanted mice is 25–35 days.

P388 leukemia was maintained by weekly serial ip passage in DBA/2 mice. A subline resistant to Act D (P388/Act D), developed at the Southern Research Institute in 1973, was maintained by implanting BDF$_1$ mice ip with 10^7 cells and treating them on Day 4 after implant with 0.2 mg/kg of Act D ip; serial passage was performed at weekly intervals. Optimal treatment with Act D usually results in the killing of at least 3 logs fewer P388/Act D cells than parental P388 cells. BDF$_1$ mice, used for all chemotherapy experiments, were implanted ip with 10^6 P388 leukemia cells harvested from the ascites fluid of leukemic passage mice.

Assessment of Antitumor Activity

Experiments involving ROS-bearing mice were initiated either on Day 12 or Days 18–20 after implant. In an attempt to treat tumors with similar growth characteristics, mice chosen for entry into experiments were selected (from among a larger pool) on the basis of their tumor sizes within specified restricted size ranges: 0.5–1.0 g for experiments begun on Day 12 and 2–3 g for experiments begun on Days 18–20. All mice were identified individually and tumor measurements were made once weekly until the termination of the experiment, ≥ 80 days after treatment. Growth of ROS tumors was estimated by converting caliper measurements to weights using the formula $W = a \times b^2/2$, where a = length in mm, b = width in mm, and w = weight in mg.

Drug toxicity was judged by the mortality patterns of tumor-bearing mice during and following treatment, and the size of the tumor at the death of the mouse (ROS does not usually metastasize). Generally, any mouse dying with a tumor smaller than the smallest tumor found among the dying untreated control mice was considered to be a drug-associated death (ie, a "drug death"). Exceptions to this rule included mice who died several weeks following treatment with growing tumors (based upon two successive weekly measurements) which never reached the size of the smallest tumor recorded among the dying untreated tumor control mice. In addition, mice dying during or shortly after treatment with large tumors (comparable to the dying untreated control mice) were considered to be drug fatalities if most of the other mice in that treatment group survived considerably longer or if the next lowest drug dose caused a substantial delay of death without any acute mortality.

Evaluation of antitumor activity against ROS was based upon several parameters: (*a*) the percentage of long-term (≥ 80 days after treatment) tumor-free survivors ("cures"); (*b*) the incidence of complete regression of measurable tumors to below palpable size (< 65 mg); (*c*) the incidence of partial regressions, defined as a reduction by $\geq 50\%$ of the tumor mass present at initiation of drug therapy; or (*d*) the increase in the median survival time (MST) of the dying mice in a treated group versus the untreated tumor-bearing control mice, also referred to as % increase in lifespan (ILS). When indicated, optimal responses obtained with Act D and analogs evaluated concomitantly were compared statistically (11).

Treatment of P388 leukemic mice began 24 hours after leukemic cell implant. Mice were observed daily for 60 days. Leukemic cell implants representing serial tenfold dilutions of each leukemic cell preparation were made concurrently in each experiment. Nonleukemic, drug toxicity control mice were also included in the leukemia study.

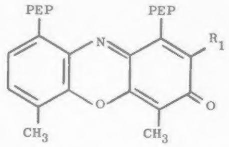
Among treated leukemic mice, the %ILS (of dying mice only) and the observed leukemic cell doubling time (based upon the titration data) were used to calculate the estimated number of leukemia cells surviving therapy (12). Antileukemic activity was evaluated on the basis of the number of leukemic cells surviving treatment and the incidence of cured mice (those alive 60 days after treatment).

All drugs were compared using the optimal therapeutic responses achieved at equitoxic doses that were less than or equal to an LD10; exceptions to this criterion are specifically stated.

Drugs

The following drugs, obtained from the Division of Cancer Treatment, NCI, Bethesda, Md, were used

TABLE 1.—Analogs of Act



Drug name	NSC No.	Position of amino acids in cyclic pentapeptide chains* 1′	2′	3′	4′	5′	R_1
Act D	3053	L-THR^2	D-VAL^2	L-PRO^2	SAR^2	L-*N*-MeVAL^2	NH_2
Act C_2	87221	L-THR^2	β-D-aILE α-D-VAL	L-PRO^2	SAR^2	L-*N*-MeVAL^2	NH_2
Act $X_0\delta$	241534	L-THR^2	D-VAL^2	β-L-aHYP α-L-PRO	SAR^2	L-*N*-MeVAL^2	NH_2
Act III	236661	L-THR^2	D-VAL^2	SAR L-PRO	SAR^2	L-*N*-MeVAL^2	NH_2
Act $Pip_1\beta$	241535	L-THR^2	D-VAL^2	L-PIP L-PRO	SAR^2	L-*N*-MeVAL^2	NH_2
3′-(4-*cis*Cl-Pro)-Act	244388	L-THR^2	D-VAL^2	4-*cis*Cl-PRO L-PRO	SAR^2	L-*N*-MeVAL^2	NH_2
Azetomicin I	244392	L-THR^2	D-VAL^2	L-AZETO L-PRO	SAR^2	L-*N*-MeVAL^2	NH_2
Azetomicin II	244393	L-THR^2	D-VAL^2	L-AZETO^2	SAR^2	L-*N*-MeVAL^2	NH_2
Act 2-hydroxy-C_3	237106	L-THR^2	D-aILE^2	L-PRO^2	SAR^2	L-*N*-MeVAL^2	OH
2-*N*-(γ-hydroxypropyl)-Act D	224135	L-THR^2	D-VAL^2	L-PRO^2	SAR^2	L-*N*-MeVAL^2	$NH(CH_2)_3OH$

*Superscript "2" refers to the presence of the amino acid on both pentapeptide chains in the position indicated. THR = threonine; VAL = valine; PRO = proline; SAR = sarcosine; *N*-MeVAL = *N*-methylvaline; aILE = *allo*-isoleucine; aHYP = *allo*-4-hydroxyproline; PIP = pipecolic acid (piperidine-2-carboxylic acid); AZETO = azetidine-2-carboxylic acid; α = substituted in the peptide chain attached to the benzenoid ring; β = substituted in the peptide chain attached to the quinoid ring.

in this study: Act D, Act C_2, azetomicin I and II, Act-2-hydroxy-C_3, 2-*N*-(γ-hydroxypropyl)-Act D, Act III, Act $X_0\delta$, Act $Pip_1\beta$, and 3′-(4-*cis*Cl-Pro)-Act. A description of how each analog differs from Act D is shown in table 1.

Each Act analog was dissolved in saline or ethanol followed by dilution with saline so that the final ethanol concentration did not exceed 2% by volume. Brown bottles were used to protect the drug solutions from light. ROS-bearing mice were injected ip with drug preparations according to their exact body weight (0.01 ml/g), whereas leukemic and drug toxicity control mice received their appropriate drug doses ip based on the average weight of the treated group.

RESULTS

Activity of Act Analogs in Mice Bearing ROS

In a pilot study (13), mice bearing established (0.5–1.0-g) ROS tumors were treated with single doses of either azetomicin I or II. Both analogs cured 100% of the mice (groups of eight to ten mice). Act D was not included in that initial study. Except for one other experiment, all subsequent testing of Act analogs was performed using mice bearing advanced, 2–3-g, ROS tumors (tables 2 and 3). Illustrations of individual tumor responses among selected groups of mice treated with particularly effective antitumor drug regimens are shown in figures 1–4.

In the first of three experiments (No. 1, table 2), single doses of each azetomicin cured more mice bearing advanced ROS than did Act D. Specifically, Act D (at an LD20) cured three of ten mice (fig 1B) and azetomicin I and II cured six of ten mice (fig 1C) and five of nine mice (fig 1D) respectively. In an attempt to confirm these initial observations, a repeat experiment (No. 2, table 2) was performed. Neither Act D nor the azetomicins displayed antitumor activity comparable to that observed in the first experiment. Similarly, Act C_2, also evaluated in this study, failed to demonstrate substantial antitumor activity (contrary to its performance in sub-

TABLE 2.—Optimal therapeutic responses of mice bearing ROS and treated with single doses of Act D and other Act analogs*

Drug name	NSC No.	Experiment No.	Dose, ip (mg/kg)	No. of— Mice	Regressions Partial	Regressions Complete	Cures	%ILS (dying mice)	Fig No.
Act D	3053	1	0.9 (LD20)	10	8	7	3	149	1B
			0.6	10	9	6	0	117	
		2	0.6	10	9	1	0	88	
		3	0.8 (LD20)	10	8	5	1	207	2A
		4	0.6	10	10	6	1	133	
		5	0.8	10	4	0	0	43	
		6	0.6 (LD20)	10	9	2	0	121	4B
Azetomicin I	244392	1	1.3	10	10	9	6	176	1C
			0.9	10	8	7	6	66	
		2	0.9	10	5	0	0	42	
		3	0.9	10	10	10	10	—	2C
Azetomicin II	244393	1	1.3	9	7	6	5	69	1D
			0.9	10	9	6	4	89	
		2	1.3	10	8	2	0	66	
		3	1.3	10	7	2	1	96	
Act C_2	87221	2	0.6	10	10	1	0	58	
		3	0.6	10	10	5	1	169	3B
		4	0.6	10	10	4	1	121	
2-*N*-(γ-hydroxypropyl)-Act D	224135	5	6.0 (LD30)	10	8	5	3	24	
		6	4.0	10	10	5	2	139	4C
Act III	236661	5	2.0 (LD20)	10	9	7	5	112	
		6	2.0	10	10	8	6	136	4D
Act $X_0\delta$	241534	5	1.0	10	10	2	0	103	
		6	1.3	6	6	3	0	125	
Act $Pip_1\beta$	241535	5	6.0	10	7	0	0	40	
		6	8.0	10	8	0	0	57	
3′-(4-*cis*Cl-Pro)-Act	244388	5	1.5	10	9	0	0	81	
		6	1.5 (LD20)	10	8	0	0	37	

*AKD2F$_1$ mice bearing sc ROS were treated when their tumors were 2–3 g in size (18–20 days after implant). All responses shown were obtained at ≤ LD10 levels, unless otherwise indicated.

TABLE 3.—Optimal therapeutic responses of mice bearing ROS and treated with multiple doses of Act D and other Act analogs

Drug name	NSC No.	Dose, ip (mg/kg/dose)	Schedule* (q14d)	No. of— Mice	Regressions Partial	Regressions Complete	Cures	%ILS (dying mice)	Fig No.
Act D	3053	0.5	× 2	10	8	4	3	157	2B
		0.4	× 3	10	9	5	0	221	
Azetomicin I	244392	0.8 (LD20)	× 2	10	9	6	5	169	
		0.6	× 2	10	9	5	3	196	
		0.6	× 3	10	9	8	8	107	2D
Azetomicin II	244393	0.8	× 2	10	1	0	0	90	
		0.6	× 3	10	0	0	0	82	
Act C_2	87221	0.5 (LD20)	× 2	10	9	8	6	119	3C
		0.4	× 3	10	9	5	2	188	3D

*AKD2F$_1$ mice bearing sc ROS were treated when their tumors were 2–3 g in size (18 days after implant). All responses were obtained at ≤ LD10 levels, unless otherwise indicated.

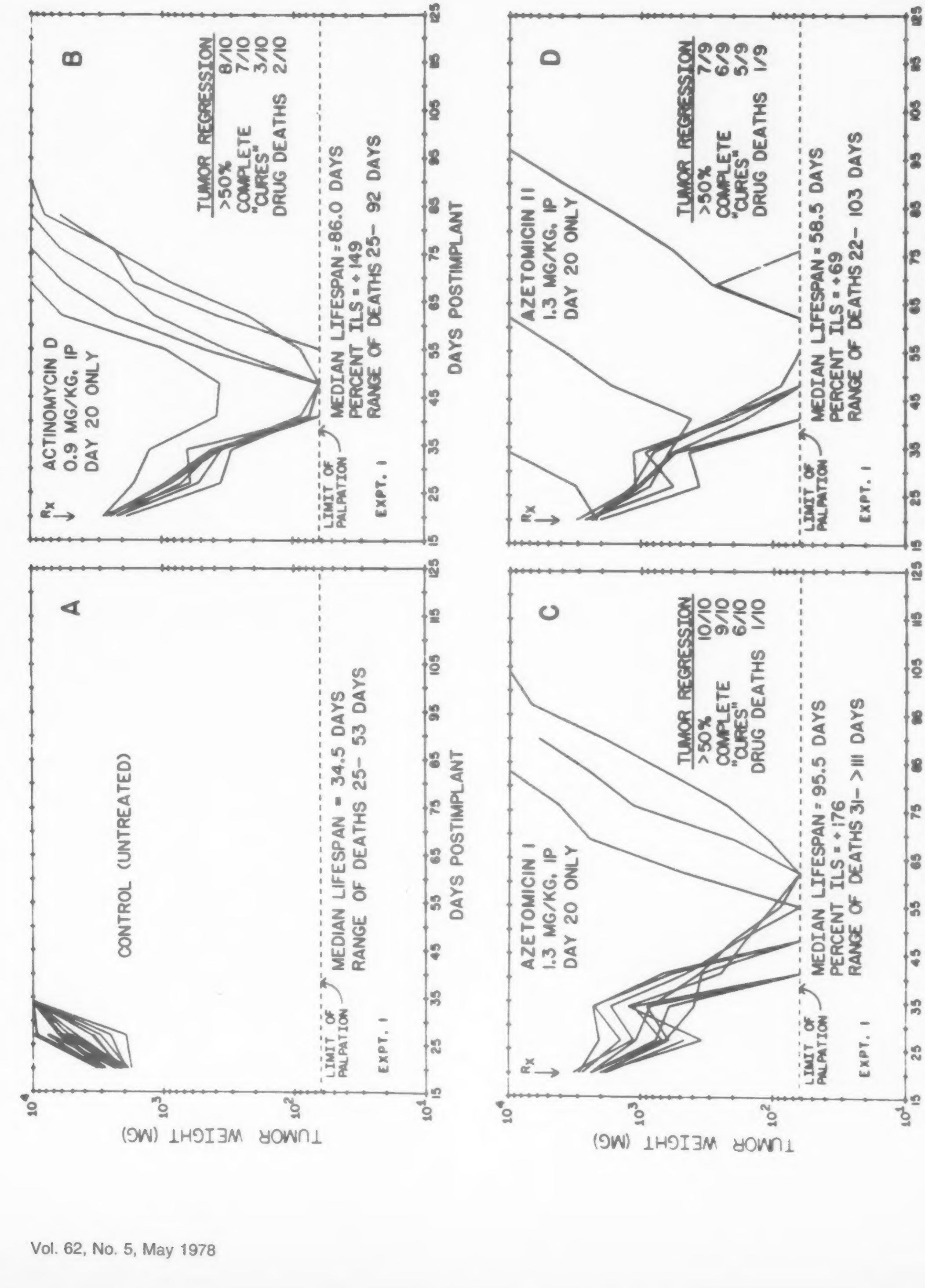


FIGURE 1.—Growth of ROS among untreated mice (A) and optimal responses obtained following treatment with Act D (B), azetomicin I (C), or azetomicin II (D).

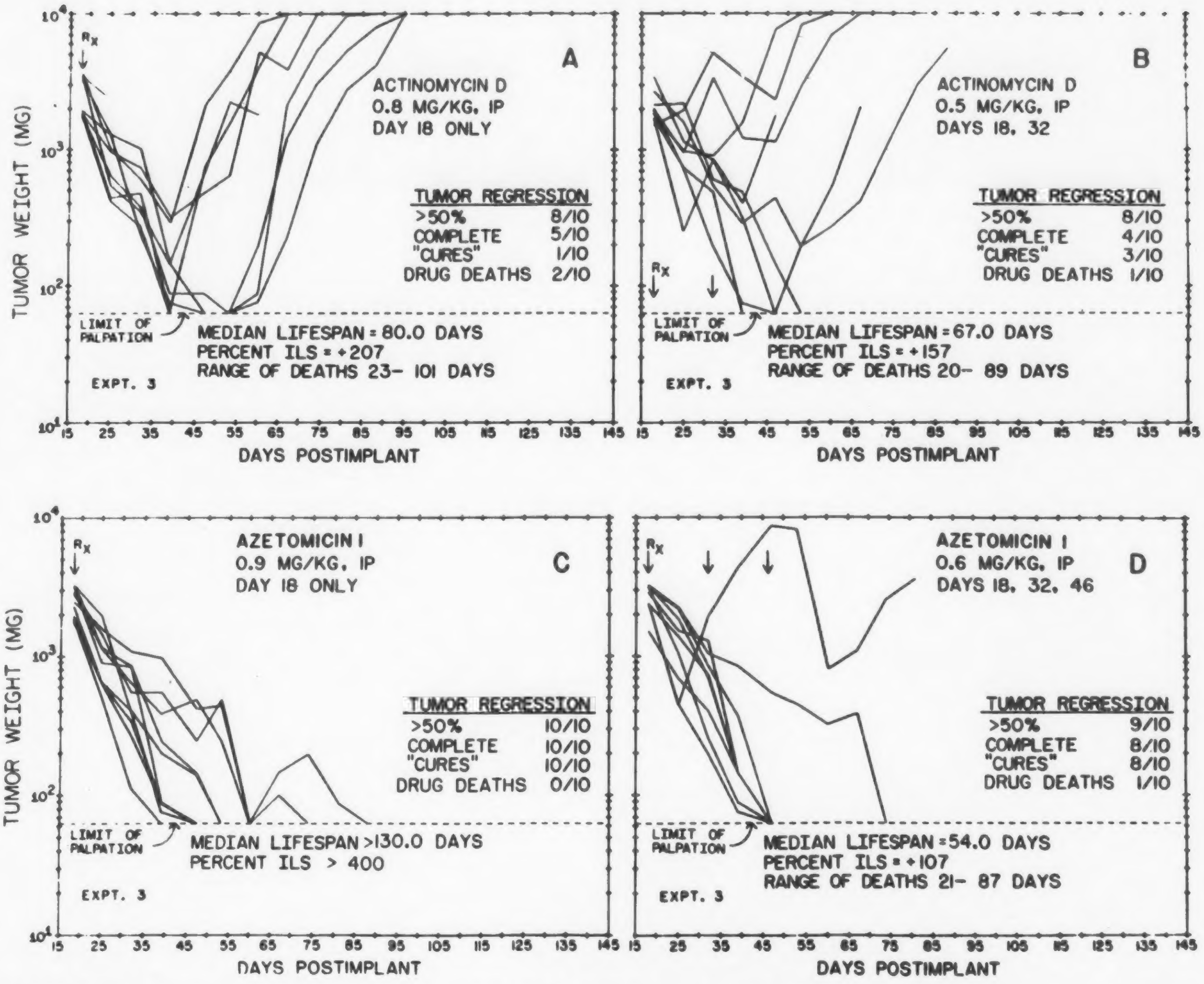


FIGURE 2.—Optimal responses of ROS-bearing mice treated with Act D, single dose (A) or q14d × 2 (B), or azetomicin I, single dose (C) or q14d × 3 (D).

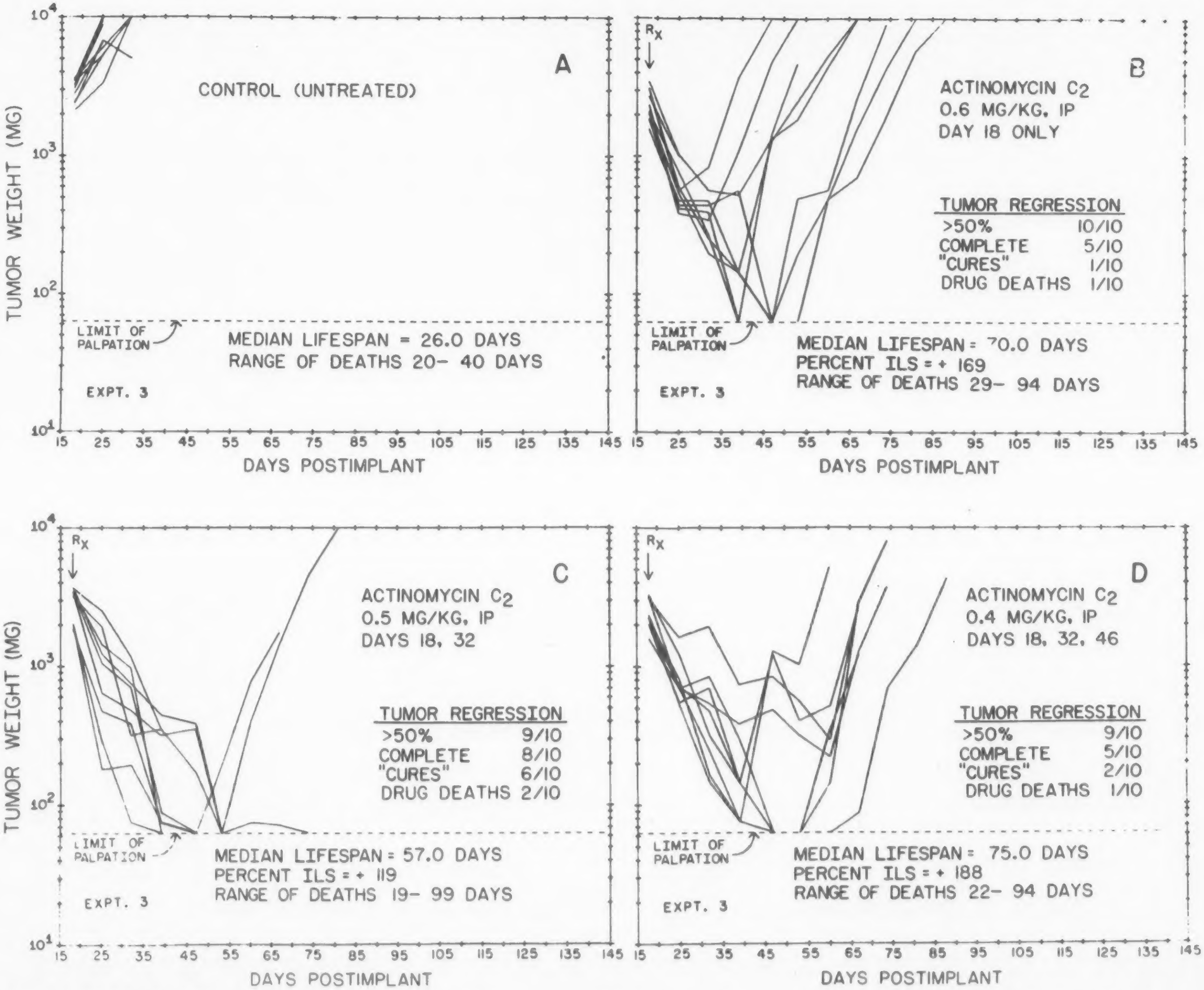


FIGURE 3.—Growth of ROS among untreated mice (A) and optimal responses obtained following treatment with Act C_2, single dose (B), q14d × 2 (C), or q14d × 3 (D).

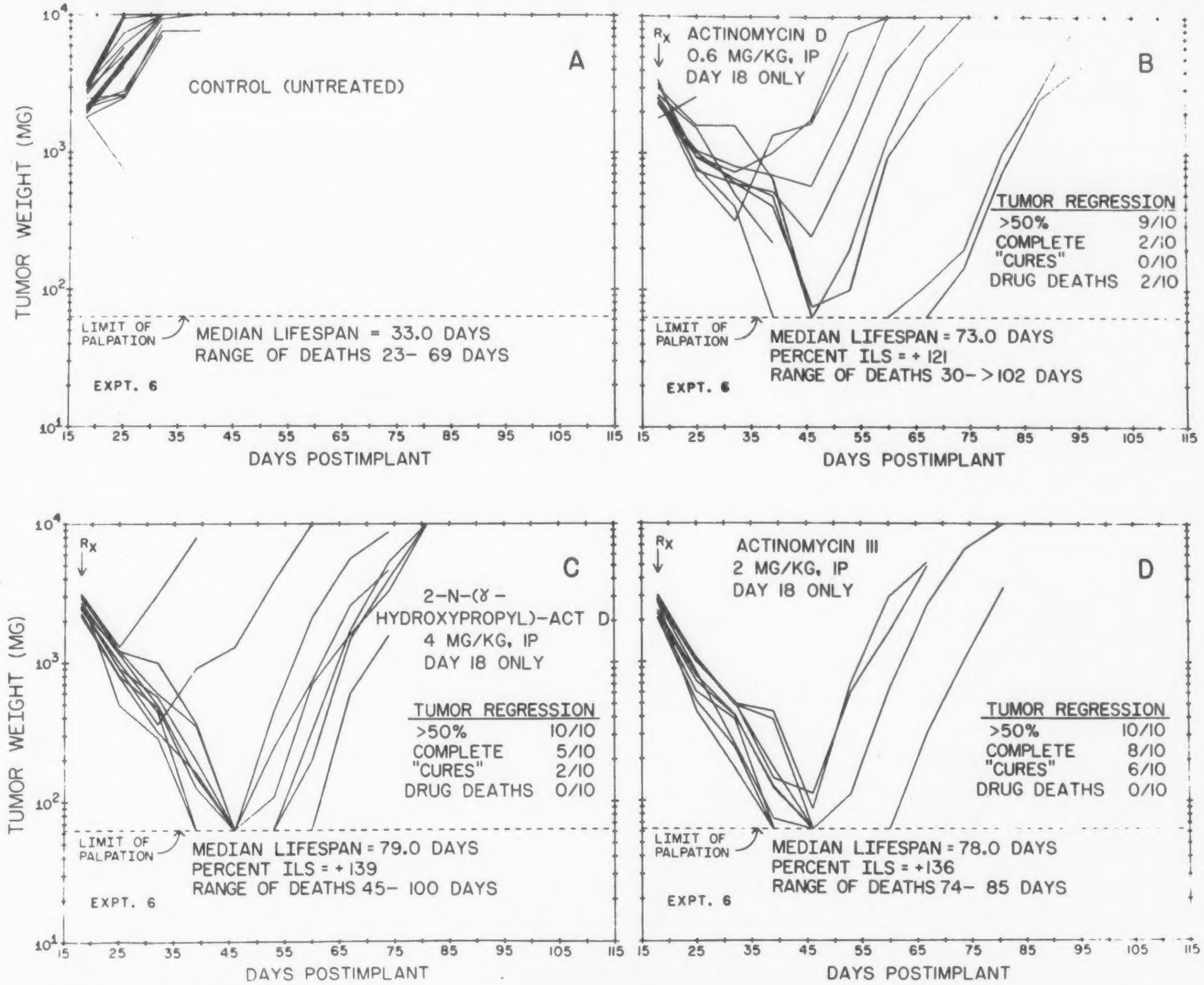


FIGURE 4.—Growth of ROS among untreated mice (A) and optimal responses obtained following treatment with Act D (B), 2-*N*-(γ-hydroxypropyl)-Act D (C), or Act III (D).

sequent experiments). None of the drugs caused more than two of ten complete regressions at acceptable (≤ LD10) levels of toxicity. The poor therapeutic responses obtained (for reasons unknown) in this experiment reflect upon the variability inherent in the test system and illustrate the necessity of including the parent compound, Act D, in each comparative study.

Additional experiments were performed using both single-dose (No. 3, table 2) and multiple-dose (table 3) treatment schedules. Azetomicin I displayed clear superiority over Act D, thus confirming the activity observed in the first experiment. The optimal therapeutic result achieved with single doses of Act D was five of ten complete regressions with one cure and a 207% ILS at an LD20 level (fig 2A). Using multiple-dose regimens, we found that Act D achieved its best response of four of ten complete regressions including three cures and a 157% ILS on a q14d × 2 schedule (fig 2B). By comparison, a single dose of azetomicin I cured ten of ten mice (fig 2C). Azetomicin I also cured three or five of ten mice (the latter at an LD20) with ILSs of 196% and 169% respectively when applied on a q14d × 2 schedule, but cured as many as eight of ten mice when a q14d × 3 treatment schedule was used (fig 2D). In this same experiment, azetomicin II achieved an optimal response of two of ten complete regressions with one cure at the highest single dose evaluated. No additional therapeutic benefit was obtained using multiple doses of the drug on q14d × 2 or × 3 schedules. Since no lethality attributed to drug toxicity was observed while using azetomicin II, better therapeutic responses might have been achieved had we tested higher doses.

The incidences of cured mice obtained in the first three experiments using optimal doses of azetomicin I and Act D were compared by statistical analysis. Despite the variable responses achieved with each drug between experiments, azetomicin I was found to have caused a significantly ($P < 0.001$) greater incidence of cured mice than did Act D. This was true regardless of whether only cures obtained with single-drug doses were compared, or whether the cure rates which followed both single- and multiple-dose treatments were considered.

Act C_2 was also tested several times in parallel with Act D (tables 2 and 3). In the first of these experiments (No. 2, table 2), neither drug displayed appreciable antitumor activity in mice bearing 2–3-g ROS tumors. In a second experiment (No. 3), both single-dose (table 2) and multiple-dose (table 3) schedules were evaluated. The optimal responses achieved with Act D have been mentioned above and are shown in figures 2A and B. By comparison, when we used a q14d × 2 schedule, an LD20 regimen of Act C_2 gave eight of ten complete regressions, including six cures, and a 119% ILS (fig 3C). Other less toxic regimens induced 50% complete regression rates with one or two of ten mice being cured and an ILS ranging from 169% to 188% (fig 3B and D). In the final experiment (No. 4, table 2), single doses of each drug again induced similar optimal therapeutic responses, ie, four to six of ten complete regressions including one cure and a 121%–133% ILS.

Additional experiments were performed to screen other Act analogs. With the exception of Act-2-hydroxy-C_3, which was tested once in mice bearing 0.5–1.0-g ROS tumors and failed to display any antitumor activity (no partial regressions), five Act analogs were evaluated twice, using single doses in mice bearing advanced 2–3-g ROS tumors. The optimal therapeutic results of those experiments are summarized in table 2, experiments 5 and 6.

In the initial experiment (No. 5), treatment with single doses of Act D caused only four of ten partial regressions. Each of the analogs produced ≥ 70% partial regression rates [albeit at an LD30 level for 2-*N*-(γ-hydroxypropyl)-Act D]. When tested again (experiment 6), Act D caused two of ten complete regressions and a 122% ILS; none of the mice were cured (fig 4B). By comparison, several of the analogs displayed superior therapeutic activity. For example, 2-*N*-(γ-hydroxypropyl)-Act D induced five of ten complete regressions, including two cures, and a 139% ILS (fig 4C), and Act III cured six of ten mice with a 136% ILS among the dying mice (fig 4D). Act $X_0\delta$ showed activity comparable to that of Act D. Finally, two other analogs, Act $Pip_1\beta$ and 3′-(4-*cis*Cl-Pro)-Act, failed to induce any complete regressions while causing only 37%–57% ILS, and were therefore inferior to Act D against ROS in this last experiment.

The complete regression and cure rates obtained in experiments 5 and 6 using optimal doses of Act III and Act D were compared statistically. The incidences of these responses achieved after therapy with Act III were significantly ($P < 0.001$) greater than those achieved after therapy with Act D.

Activity of Act Analogs in Mice Bearing P388 or P388/Act D Leukemia

Both azetomicin I and II were tested in parallel with Act D against P388 leukemia and a subline in which the cells were resistant to Act D (P388/Act D). As shown in table 4, mice implanted with 10^6 P388/0 leukemia cells (the parental line) responded well to both azetomicins, with 4–6 logs fewer leukemia cells remaining after the q4d × 3 treatment and 30%–43% of the mice cured. Act D also demon-

TABLE 4.—Optimal therapeutic responses of mice bearing P388/0 or P388/Act D to azetomicin I and II (treatment given ip, Days 1, 5, and 9)

Drug name	NSC No.	Dose (mg/kg/dose)	10^6 cells, ip	No. of mice alive on Day 60/total treated	Median (range)		Approximate No. of leukemic cells alive at end of treatment*
					Day of death (dying mice only)	%ILS	
None		Leukemia control	P388/0	0/20	11.0 (8–14)	—	—
Act D	3053	0.3		5/10	25.0 (6–36)	127	1.6×10^4 (0.7)
Azetomicin I	244392	0.6		3/10	37.0 (13–44)	236	ca 1 (1.2)
Azetomicin II	244393	0.6		3/7	30.0 (21–34)	172	2.7×10^2 (0.8)
None		Leukemia control	P388/Act D	0/20	11.0 (10–15)	—	—
Act D	3053	0.3		0/10	18.0 (13–21)	63	4.2×10^6
Azetomicin I	244392	0.6		0/10	16.0 (12–20)	45	1.8×10^7
Azetomicin II	244393	0.6		0/8	15.5 (13–18)	40	2.5×10^7

*Based on median day of death of dying mice (12). Figures in parentheses are based on % survivors.

strated marked antileukemic activity, curing five of ten mice at the optimum dose level. These data indicate that the three drugs, azetomicin I and II and Act D, have similar activity against ip implanted P388 leukemia.

Historically, Act D kills about 3 logs fewer P388/Act D leukemia cells than it does parental P388/0 cells. In the experiment shown in table 4, only about a 2-log difference in cytotoxic effect was found, based on lifespan of dying mice, but a higher degree of resistance was suggested based on the comparative incidences of surviving mice after Act D therapy. According to data from this experiment, it was also apparent that the P388 leukemia resistant to Act D was cross-resistant to azetomicin I and II.

DISCUSSION

Because ROS is a solid murine tumor that has been shown to be markedly sensitive to Act D (7–10), it was used for evaluating Act analogs. In addition, we included P388 leukemia in our studies because this tumor system is used by the NCI for primary screening of potential anticancer drugs and it too was reported to be sensitive to Act D (9,10). It has been reported (14) that a single nonlethal dose of Act D was curative in mice bearing ROS at a stage where repeated daily doses for 7 days were not curative (despite a total dose equal to the LD10 in normal mice). We therefore relied primarily upon single doses for screening analogs against ROS in the present studies. Against P388 leukemia (both Act D-sensitive and -resistant sublines), we used a q4d × 3 schedule which our experience had shown to be most effective.

Two Act analogs, azetomicin I and Act III, caused therapeutic responses among mice bearing advanced ROS tumors which, although variable, were nevertheless indicative of antitumor activities greater than was found using Act D. In one instance, a single dose of azetomicin I cured all the treated mice; this effect had never been observed following Act D therapy (including all treatment schedules evaluated) against this stage of ROS tumor progression in 20 experiments conducted over a 3-year period. It is interesting that both azetomicin I and Act III differ from Act D in the replacement (with either azetidine-2-carboxylic acid or sarcosine respectively) of one proline residue from the third position in one of the pentapeptide side chains of the molecule (15,16).

Several other analogs, Act C_2, 2-*N*-(γ-hydroxypropyl)-Act D, Act $X_0\delta$, and azetomicin II, displayed antitumor activity in ROS-bearing mice comparable to, or on single occasions superior to, that achieved using Act D. Goldin and Johnson (10) have reported that Act C_2 was comparable to Act D when used to treat mice bearing P388 leukemia. Finally, Act Pip$_1\beta$ and 3′-(4-*cis*Cl-Pro)-Act were comparable to, and Act-2-hydroxy-C_3 inferior to, Act D in anti-ROS activity.

From the P388 leukemia experiments, we can ascertain that both azetomicins displayed antileukemic activity comparable to Act D. Moreover, a subline of P388 leukemia that was resistant to Act D was found to be cross-resistant to both azetomicins. This last finding was particularly disappointing but expected.

Although ROS is quite susceptible to the Act analogs, as well as many other clinically useful anticancer drugs (8), the activity of any promising analog should be evaluated in numerous model tumor systems. On the basis of the data presented, further study with azetomicin I and Act III is warranted before any decision is made regarding clinical trials.

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Distribution and Antitumor Activity of Adriamycin Given in a High-Dose and a Repeated Low-Dose Schedule to Mice[1,2]

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SUMMARY

Experimental studies on the distribution of adriamycin (AM) under different treatment conditions and possible correlations between tissue and plasma levels and chemotherapeutic activity are discussed. C57BL/6J mice bearing im Lewis lung carcinoma and (C3H × O2O)F_1 mice bearing mammary carcinoma were injected iv with AM at a single dose of 15 mg/kg or with the same total amount of drug administered in spaced doses of 3.75 mg/kg for 4 consecutive days. In the two experimental systems studied, the drug reached approximately the same value in the tumor and spleen with both types of treatment, but with the 3.75-mg/kg × 4 schedule much lower AM concentrations were observed in the heart than with the single high-dose treatment. The therapeutic activity of the two treatments also differed: the antitumor and antimetastatic effect was the same in the two tumor systems, but with the 3.75-mg/kg × 4 schedule, increased survival and somewhat lower toxicity were observed. Daunorubicin, tested in the mammary carcinoma system with the two schedules of treatment, behaves very similarly to AM in terms of both distribution and chemotherapeutic effect.

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Among the antitumor antibiotics, adriamycin (AM) has gained increasing importance because of its effectiveness, particularly against a variety of solid tumors (1–3). As with other chemotherapeutic agents, however, toxicity is a limiting factor in AM therapy. Alopecia, stomatitis, myelosuppression, fever, nausea, and phlebitis are some of the toxic effects of this drug in man (1,3,4). The greatest limitations to its clinical use, though, are its strong cardiotoxic effects which consist of electrographic alterations and congestive heart failure (3–9).

Various studies are currently underway to clarify the mechanisms involved in AM heart toxicity. In the framework of this investigation, pharmacokinetic studies of the compound may provide useful information for improving dose schedules.

This report presents measurements of plasma and tissue levels of AM, particularly in tumor and heart, in two experimental tumor systems of the mouse after injection of either a single high dose or repeated daily treatments to reach the same total dose; data on the in vivo chemotherapeutic activity of these treatment schedules are also supplied.

Both AM and daunorubicin (DR) reach considerably lower levels in the heart after repeated low doses than after a single high dose; the possibility is discussed that such differences in drug distribution, particularly in the heart, might be related in some way to the superior therapeutic activity observed with the repeated treatment schedule.

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MATERIALS AND METHODS

Animals and Tumors

Male C57BL/6J mice (22 ± 2 g in body weight), obtained from Charles River (Italy), received an im transplant of 2 × 10^5 viable cells of the syngeneic Lewis lung carcinoma (3LL), maintained by im passage in the same strain every 2 weeks and known to give rise to macroscopic metastases to the lung (10).

A group of animals bearing tumors weighing about 2 g (11–14 days after implantation) were treated as described below and used for pharmacokinetic studies, and another group received the same treatment for evaluation of drug antitumor activity. On Day 25 after tumor transplantation, this second group was killed and tumor weights were recorded. A previously described procedure (11) was followed for measuring lung metastases. A third group of animals was used to record the survival time.

$(C3H \times O2O)F_1$ male and female hybrid mice (30 ± 2 g in body weight) bearing spontaneous mammary carcinomas were obtained from the International Center for the Provision and Study of Tumor Bearing Animals (Amsterdam, Netherlands). These carcinomas were the result of treating castrated male mice at an early age with estrogens added to their drinking water and of force breeding female mice (12–14).

As for the 3LL, animals bearing mammary tumors of 2 ± 0.3 g were used either for pharmacokinetic studies or for evaluation of drug antitumor activity. Inhibition of tumor growth was determined by measuring tumor diameters with vernier calipers; values in millimeters were then converted to weight measurements using the spheroid formula $w = \frac{ab^2}{2}$, where a is the length and b the width of the tumor, assuming density equal to 1.

For both tumor systems, deaths among treated mice occurring before the death of the first control mouse were considered toxic deaths and were not included in the evaluation.

The animals were housed in Makrolon cages (20 × 20 × 13 cm) at a room temperature of 22°C and relative humidity of about 60%, with free access to food and water during the experiments.

Drugs and Experimental Design

AM and DR were freshly dissolved in saline before iv injection. Drug levels in serum were measured as described by Finkel et al (15); according to this method, recovery was 90% and sensitivity was 0.05 μg/ml. For the tumor and other tissues, extraction with $AgNO_3$ was carried out to release AM and DR bound to DNA and RNA and to precipitate proteins (as recommended by Schwartz [16]), and the supernatant was subsequently extracted with *n*-butyl alcohol. Recovery was 75% and sensitivity was 1 μg/g of tissue. Readings were taken in an Aminco Bowman Spectrofluorometer at 470 mμ excitation and 569 mμ emission.

For pharmacokinetic studies, the groups receiving the repeated dose of 3.75 mg/kg iv were treated for 4 consecutive days when the tumor weight had reached approximately 2 g; this was from Day 11 after tumor transplantation for 3LL and from Day 0 for mammary carcinoma. On Days 14 and 4 additional groups were given either 3.75 or 15 mg/kg × 1, respectively.

Experimental conditions were the same in the group undergoing evaluation of antitumor activity, except that in addition, for the 3LL (which is a fast growing tumor), in order to improve the comparison between the single high-dose and the repeated low-dose schedules, a further group was given 15 mg/kg on Day 11, ie, at the beginning of the repeated treatment schedule.

Data Analysis

The areas under the tumor growth versus time curve (AUC_1) and the areas under the AM concentration versus time curve (AUC_2) were calculated by trapezoidal integration. Statistical significance of the differences between values of controls and treated groups was assessed by Duncan's test or Student's t test. Fischer's exact test was employed for percent values and the Quantile test ($P^{\pm} = 0.50$) for median survival times.

RESULTS

3LL

The first experiment was designed to obtain information on the concentrations of AM equivalents in the 3LL tumor after iv treatment with graded doses of AM and the corresponding tumor inhibitory effect. Drug levels (table 1) measured up to 72 hours show peak values ranging between 1 and 3–4 μg/g of tissue and AUC_2 values progressively increasing, depending on the dose, although the AUC_2 of the 18.75-mg/kg dose is only slightly greater than that of the 15-mg/kg dose. Correspondingly, a dose-response effect is observed in terms of tumor growth inhibition during the 72 hours.

The lowest dose employed (3.75 mg/kg) was effective even though under these conditions the drug

TABLE 1.—Tumor growth inhibition by graded single doses of AM and corresponding drug levels in im 3LL*

Treatment dose (mg/kg iv)	Peak level (μg/g ± SE)	AUC_2 after 72 hrs (μg/g × min ± SE)	Toxic deaths in 72 hrs (%)	Tumor weight inhibition	
				g ± SE	%
—	—	—	—	4.21 ± 0.2	—
3.75	< 1	< 1	0	3.09 ± 0.1†	27.6†
7.5	1.7 ± 0.2	2076 ± 228	0	2.87 ± 0.3†	31.7†
11.25	2.2 ± 0.1	7320 ± 827	0	2.79 ± 0.3†	33.8†
15	3.0 ± 0.6	10,792 ± 738	17	2.45 ± 0.2†	41.8†
18.75	4.9 ± 0.8	10,995 ± 1651	50	2.07 ± 0.1†	50.8†

*Drug levels in the tumor were measured 0.5, 1, 3, 7, 24, and 72 hrs after iv injection of AM. C57BL/6J mice transplanted im 14 days before with 2 × 10^5 3LL tumor cells were treated with AM and killed 72 hrs later to measure tumor weights.

†$P < 0.01$ vs controls.

TABLE 2.—Serum and tissue levels of AM equivalents after iv injection of 3.75 mg/kg × 4 or 15 mg/kg × 1 in im 3LL-bearing mice*

	Peak level† (μg/ml or g ± SE)		AUC_2 after 24 hrs (μg/ml or g × min ± SE)	
	3.75 × 4	15 × 1	3.75 × 4	15 × 1
Serum	8.2 ± 0.9	23.2 ± 1.4‡	18 ± 2	283 ± 62
Tumor	2.0 ± 0.2	2.3 ± 0.3	2652 ± 343	2520 ± 321
Heart	8.0 ± 0.1	24.9 ± 0.5‡	5919 ± 540	17,121 ± 645‡
Spleen	28.0 ± 2.7	37.2 ± 4.2	32,478 ± 4081	51,415 ± 5082

*C57BL/6J mice bearing im 3LL weighing 1.9–2.3 g were used.

†Time of peak concentration is the same as in fig 1.

‡$P < 0.01$ vs 3.75-mg/kg × 4 group.

does not reach detectable levels in the tumor. When, however, two injections of 3.75 mg/kg were given at an interval no > 48 hours, AM levels in the tumor enter the sensitivity range of the method (unpublished data).

AM distribution studies were then made in 3LL im transplanted mice after a single 15-mg/kg iv dose or after a 3.75-mg/kg dose given singly or for 4 consecutive days. The 15-mg/kg dose was selected as being the highest dose (under our conditions) able to give rise to a marked tumor growth inhibition (> 40%) with a reasonable toxicity (17%). In an attempt to reduce the toxicity observed, the same total drug amount of 15 mg/kg was then fractionated in four injections of 3.75 mg/kg, a dose which when administered at an interval of 24 hours is expected, as mentioned above, to determine drug accumulation in the tumor. Figure 1 shows that with the 3.75-mg/kg × 1 schedule, AM concentrations were not measurable in the primary tumor, whereas in the heart, the drug reached 8.4 μg/g but was no longer evident at 24 hours; in the spleen, AM concentrations remained constant around 6 μg/g throughout the 24-hour period. When AM levels were measured at different times after the last injection in the 3.75-mg/kg × 4 schedule, drug concentrations found in the primary tumor were around 2 μg/g; levels in the heart were practically the same as those present with the 3.75-mg/kg × 1 treatment.

Comparing these results with those after the 15-mg/kg treatment, no marked differences were seen in the levels reached in the tumor and spleen, but the 3.75-mg/kg × 4 schedule gave drug concentrations in the heart approximately three times lower (8 μg/g as opposed to 25 μg/g for the high-dose schedule).

Table 2 summarizes the peak levels and corresponding AUC_2 values in these experiments after the single high-dose and repeated low-dose treatment. Again, the AM levels in the tumor and spleen are approximately equal with both treatments, but much higher AM concentrations are observed in the heart after a single high dose.

The chemotherapeutic activity of the two treatments is shown in table 3. The effects on the weight

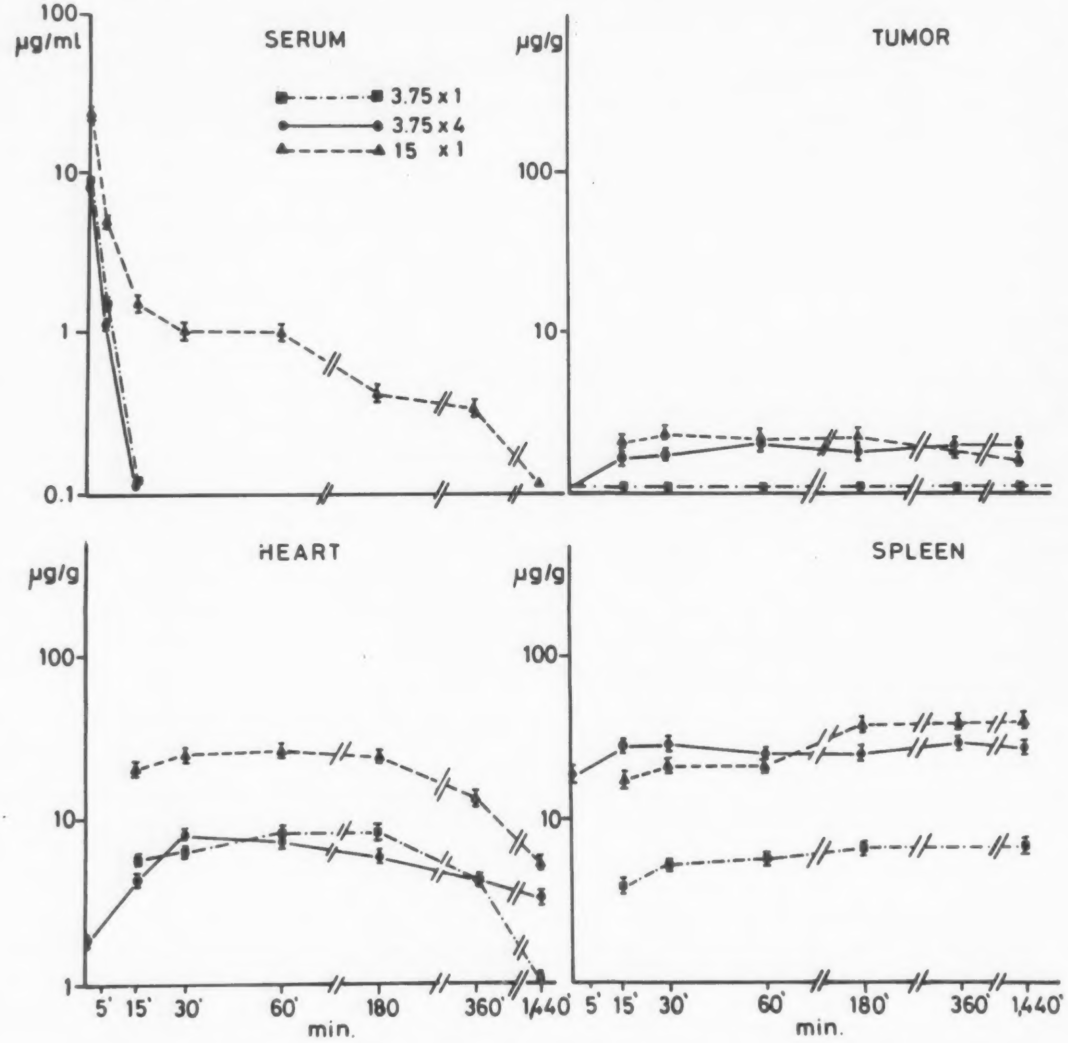

FIGURE 1.—AM distribution in C57BL/6J mice bearing im 3LL carcinoma after injection of 3.75 mg/kg iv × 1 (■ - · - · - ■), 3.75 mg/kg iv × 4 (● —— ●), or 15 mg/kg iv × 1 (▲ - - - - ▲). Drug determination on Day 14 after im transplant of 2 × 10^5 tumor cells.

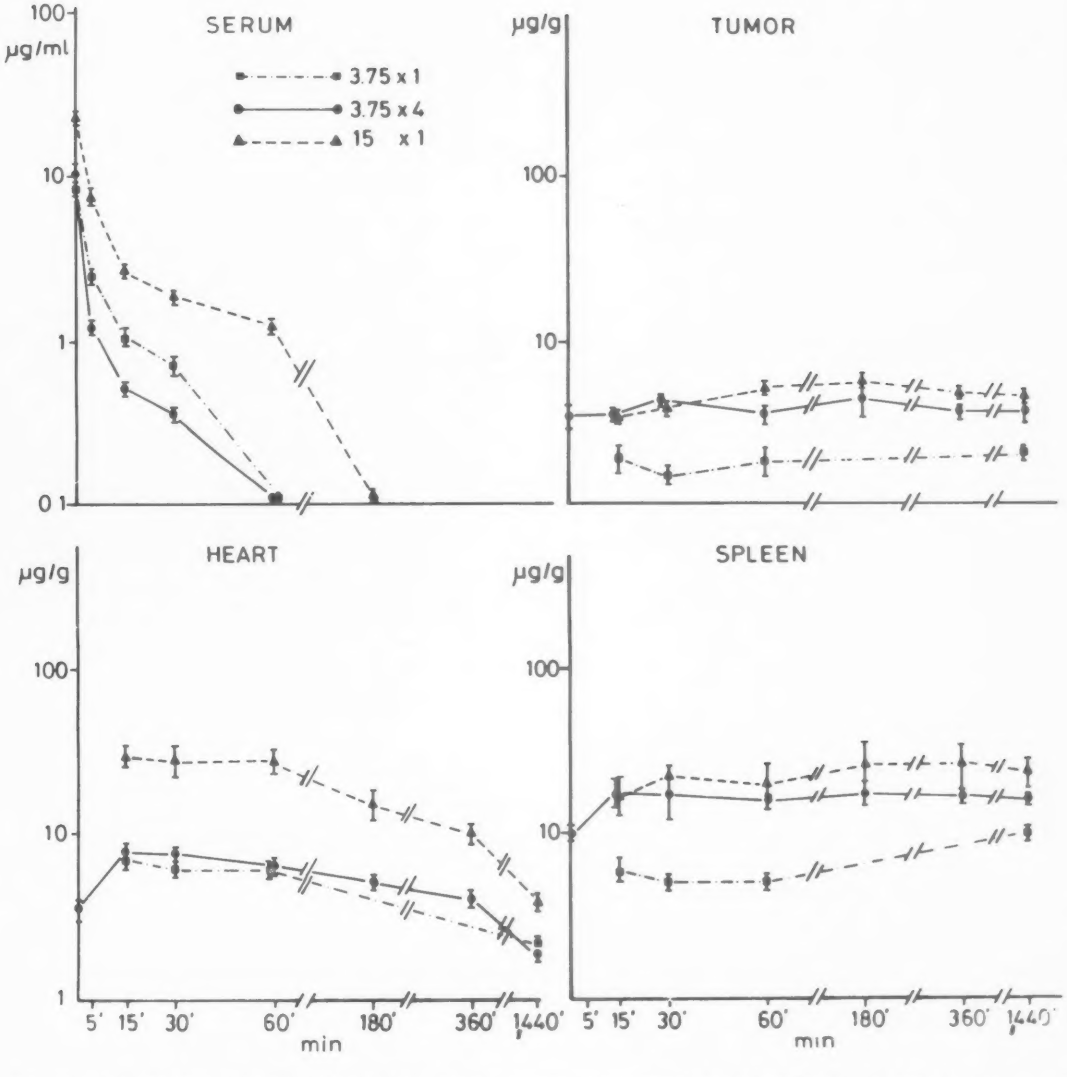

FIGURE 2.—AM distribution in (C3H × O2O)F$_1$ mice bearing mammary carcinoma after injection of 3.75 mg/kg iv × 1 (■ - · - · - ■), 3.75 mg/kg iv × 4 (● —— ●), or 15 mg/kg iv × 1 (▲ - - - - ▲).

TABLE 3.—AM antitumor activity on im 3LL after iv injection of 15 mg/kg × 1 or 3.75 mg/kg × 4*

Treatment (mg/kg iv)	Toxic deaths (%)	Primary tumor weight (g ± SE)	Metastases-free mice (%)	Metastases		Survival		Long-term survivors (%)†
				No. per mouse ± SE	Weight (mg) per mouse ± SE	Mean (days ± SE)	Median (days)	
—	0	9.4 ± 0.3	0	27.0 ± 2.7	168.9 ± 35.5	29 ± 1	28	—
3.75 × 4 (Days 11–14)	10	7.9 ± 0.5‡	25	2.0 ± 0.3§	3.2 ± 1.4§	36 ± 1.6‡,¶	36‡,¶	57‡,¶
15 × 1 (Day 11)	35	7.6 ± 0.3‡	18	2.6 ± 0.6§	12.1 ± 4.7§	30 ± 1.3	31	0
15 × 1 (Day 14)	35	7.8 ± 0.3‡	18	2.6 ± 0.3§	1.4 ± 0.4§	32 ± 1.4	32	18

*Each group consisted of 10 mice; antitumor and antimetastatic activity was evaluated 25 days after im transplantation of 2 × 10^5 tumor cells in C57BL/6J mice. Day of tumor transplant was considered Day 0.

†% of treated animals surviving after the day the last control died.

‡$P < 0.05$ vs controls.

§$P < 0.01$ vs controls.

¶$P < 0.05$ vs 15-mg/kg × 1 (Day 11) group.

TABLE 4.—Serum and tissue levels of AM equivalents after iv injection of 15 mg/kg × 1 or 3.75 mg/kg × 4 in mammary carcinoma-bearing (C3H × O2O)F_1 mice*

	Peak level† (μg/ml or g ± SE)		AUC_2 after 24 hrs (μg/ml or g × min ± SE)	
	3.75 × 4	15 × 1	3.75 × 4	15 × 1
Serum	10.0 ± 1.3	22.3 ± 1.0	38 ± 4	187 ± 18
Tumor	4.2 ± 1.0	5.4 ± 1.3	5311 ± 926	6684 ± 1101
Heart	7.7 ± 0.4	29.8 ± 2.7‡	4964 ± 412	13,754 ± 1733‡
Spleen	17.6 ± 0.7	26.1 ± 3.6	23,808 ± 2663	34,957 ± 2798

*Tumor weight when treatment began was 1.8–2.1 g.

†Time of peak concentration is the same as in fig 2.

‡$P < 0.01$ vs 3.75-mg/kg × 4 group.

of the primary tumor and on the weight, takes, and number of pulmonary metastases were practically the same with both the 3.75-mg/kg × 4 schedule (given from Days 11 to 14) and the 15-mg/kg × 1 treatment (given on either Day 11 or Day 14 after tumor transplantation); however, the single high-dose treatment was accompanied by a higher proportion of toxic deaths and a somewhat shorter survival time. It is also interesting to note that almost 60% of the animals treated with the 3.75-mg/kg × 4 schedule, as compared to 0 or < 20% of the 15-mg/kg × 1 group, were still alive 35 days after treatment, when all controls were dead.

Mammary Carcinoma

Measurements of serum and tissue levels of AM equivalents in mice bearing mammary carcinoma treated with the same schedules as the 3LL system (fig 2 and table 4) indicate that the 15-mg/kg × 1 and the 3.75-mg/kg × 4 schedules both gave practically equivalent drug levels during the first 24 hours in the tumor and spleen, whereas the single high-dose treatment gave significantly higher concentrations in the serum and heart. In the heart, the concentrations were approximately three times higher (about 30 μg/g with the 15-mg/kg × 1 treatment and < 8 μg/g in the 3.75-mg/kg × 4 group). Again, as for the 3LL, the 3.75-mg/kg × 1 treatment gives AM levels in the heart comparable to those following the repeated schedule, indicating that no drug accumulation should occur in these conditions.

However, at variance with the first tumor system, a single dose of 3.75 mg/kg results in drug levels in the tumor of about 2 μg/g; the 15-mg/kg × 1 and 3.75-mg/kg × 4 schedules give about twice as much AM (4–5 μg/g) as in 3LL tumor tissue.

Figure 2 also shows that serum levels following multiple treatments are somewhat lower than after a single 3.75-mg/kg treatment, suggesting that pre-

TABLE 5.—AM antitumor activity on mammary carcinoma after iv injection of 15 mg/kg × 1 or 3.75 mg/kg × 4*

Treatment (mg/kg iv)	Toxic deaths (%)	Tumor weight AUC_1 (g × 24 days ± SE)	Survival		Long-term survivors (%)†
			Mean (days ± SE)	Median (days)	
—	—	74 ± 12	42 ± 6	43	—
3.75 × 4	0	39 ± 6‡	69 ± 5‡,§	62§,¶	42¶
15 × 1	20	37 ± 6‡	49 ± 4	53	18

*Each group consisted of 10 mice; $(C3H \times O2O)F_1$ mice bearing mammary carcinoma of about 2 g were used. Day of the 1st 3.75-mg/kg dose was considered Day 0.

† % of treated animals surviving after the day the last control died.

‡$P < 0.01$ vs controls.

§$P < 0.05$ vs 15-mg/kg × 1 group.

¶$P < 0.05$ vs controls.

TABLE 6.—Serum and tissue levels of DR equivalents after iv injection of 15 mg/kg × 1 or 3.75 mg/kg × 4 in mammary carcinoma-bearing mice*

	Peak level† (μg/ml or g ± SE)		AUC_2 after 24 hrs (μg/ml or g × min ± SE)	
	3.75 × 4	15 × 1	3.75 × 4	15 × 1
Serum	< 0.05	2.7 ± 0.6 (15 mins)	< 0.05	924 ± 89
Tumor	3.6 ± 0.4 (6 hrs)	7.5 ± 1.2 (6 hrs)	2550 ± 337	11,822 ± 1609‡
Heart	10.5 ± 0.8 (15 mins)	65.2 ± 8.6‡ (15 mins)	3465 ± 275	22,643 ± 3671‡
Spleen	19.0 ± 3.4 (3 hrs)	55.8 ± 3.6‡ (6 hrs)	16,247 ± 1767	57,519 ± 5089‡

*$(C3H \times O2O)F_1$ mice bearing mammary carcinoma weighing 1.7–2 g were used.

†Nos. in parentheses = time of peak concentration; initial sampling schedule started from 15 mins.

‡$P < 0.01$ vs 3.75-mg/kg × 4 group.

vious AM treatments may affect in some way the elimination rate of subsequent drug administrations. This indication should however be confirmed in further experiments.

The chemotherapeutic activities of the high-dose and the repeated low-dose schedules of AM on mammary carcinoma are compared in table 5; the results are similar to those described for the 3LL system. The 3.75-mg/kg × 4 AM treatment (the day of the first injection being taken as Day 0, when the primary tumor weight was in the 1.7–1.9-g range) led to the same reductions in tumor weight as with the 15-mg/kg × 1 schedule. The toxicity observed with the repeated schedule seems lower (no early deaths were observed) than with the single high-dose schedule (20% deaths). This may have contributed to the better survival in the 3.75-mg/kg × 4 group; the mean and median survival times were significantly longer than with the 15-mg/kg × 1 treatment.

DR Studies

The two schedules described above were also compared employing DR instead of AM in mammary carcinoma-bearing $(C3H \times O2O)F_1$ mice. DR concentrations, expressed as DR equivalents, were measured in serum and tissues under the same experimental conditions used for AM (table 6). Results obtained with this agent, administered as a single high dose of 15 mg/kg × 1 or as repeated low doses of 3.75 mg/kg × 4, were partially different from those seen for AM; a single dose of 15 mg/kg of DR produced significantly higher levels than the repeated schedule not only in the heart (where there was an approximately sixfold difference in peak level and AUC_2 value) but also in the spleen and tumor. No drug was detectable in serum in the 3.75-mg/kg × 4 group.

The in vivo activity of these DR schedules is summarized in table 7. The reduction in tumor mass

TABLE 7.—DR antitumor activity on mammary carcinoma after iv injection of 15 mg/kg × 1 or 3.75 mg/kg × 4*

Treatment (mg/kg iv)	Toxic deaths (%)	Tumor weight AUC_1 (g × 22 days ± SE)	Survival		Long-term survivors (%)†
			Mean (days ± SE)	Median (days)	
—	—	111 ± 18	32 ± 4	31	—
3.75 × 4	10	41 ± 5‡	51 ± 5‡,§	50	75‡
15 × 1	20	44 ± 10‡	40 ± 4	36	25

*Each group consisted of 10 mice; (C3H × O2O)F_1 mice bearing mammary carcinoma of about 2 g were used. Day of the 1st 3.75-mg/kg dose was considered Day 0.

† % of treated animals surviving after the day the last control died.

‡$P < 0.01$ vs controls.

§$P < 0.05$ vs 15-mg/kg × 1 group.

is similar after both treatments, but the repeated schedule seems to prolong the survival time of treated mice more than the 15-mg/kg schedule as compared to controls. In fact, with toxicity approximately equal in the two groups, 75% of the 3.75-mg/kg × 4 group survived > 42 days, ie, the day of the last control death, as compared to 25% long-term survivors in the 15-mg/kg × 1 group.

DISCUSSION

This study presents additional data indicating that the pattern of AM distribution may vary considerably depending on the treatment schedule employed. Dose-dependent AM levels were found in plasma and tissues from C57BL/6J mice bearing im 3LL after iv injection of two doses, both active in inhibiting tumor growth (3.75 or 15 mg/kg). One injection of 3.75 mg/kg does not result in measurable levels of AM in serum and tumor and seems to disappear from the heart within approximately 24 hours; if repeated at an interval of at least 24 hours, it becomes detectable in the tumor without accumulating in the heart. When 3.75 mg/kg is injected daily for 4 consecutive days, drug concentrations in the heart after the last dose are almost the same as after the first injection. Comparison of distribution of AM in plasma and tissues after four doses of 3.75 mg/kg or after a single iv push of 15 mg/kg, shows that the drug levels reached in the tumor and spleen are approximately equal, but the AM concentration in the heart is markedly lower after the repeated low doses.

The repeated low doses have antitumor activity similar to that of the single 15-mg/kg dose in terms of primary and metastatic burden reduction, but seem to increase survival. Similar conclusions can be drawn from the studies in (C3H × O2O)F_1 mice bearing a spontaneous mammary carcinoma treated with AM as above. Again, tumor and spleen drug levels did not differ significantly but AM concentrations in the heart were markedly lower in the 3.75-mg/kg × 4 group than in the high-dose group.

Comparison of the in vivo chemotherapeutic effect reveals even more clearly than with the 3LL the superiority of the repeated schedule over the single-dose treatment; tumor growth was inhibited to the same extent, but survival of the 3.75-mg/kg × 4 group was significantly longer and toxicity was less.

DR, also tested in the mammary carcinoma system, behaves similarly to AM with regard to both distribution and chemotherapeutic effect after single high-dose or repeated low-dose treatments.

Our findings that AM reaches lower drug levels in the hearts of laboratory animals after repeated low doses than after a single high dose are in agreement with a report by Arena et al (17); however, to what extent the different amounts of AM or DR in the heart may induce different cardiotoxicity in vivo, possibly contributing to different general toxicity of these agents, has yet to be shown. However, it seems reasonable to assume that, since the therapeutic and toxic effects of drugs may be related to the amount to which tissues are exposed, treatments giving rise to lower drug concentrations in the heart may be associated with lower in vivo cardiotoxicity.

That cardiotoxicity may be a contributing factor to AM-induced lethality in man has been suggested by previous studies (18) and specifically in mice by Rosenoff et al (19).

The dose and/or schedule dependence of anthracycline-induced cardiomyopathy has been widely described in clinical and experimental studies (17,20–22) and dose limitation is mandatory in clinical use. However, in our experiments, measurement of AM and DR levels does not discriminate between the unchanged drugs and their metabolites. Knowledge of the relative quantities of metabolites in the heart may clarify the significance of the concentrations found. The cardiotoxicity of the anthracycline antibiotics has been ascribed to the daunosamine moiety of the molecule (23), and it has also been reported that aglycones, which are AM and DR metabolites, may cause greater cardiotoxicity than the parent drug (7,24). Altering the levels of AM and/or its metabolites in the heart by changing the dose schedule might therefore prove to be an important way of limiting cardiotoxicity.

With regard to the differences in therapeutic effect displayed by AM and DR, it should be noted that, besides the heart, other target organs for toxicity such as the immune and hematologic systems may be important in determining the final response. Although more intensive studies are needed on the relative general toxicity of these drugs, our findings suggest that a better therapeutic effect may be obtained with close repeated doses than with a single high-dose iv push, as is the more common clinical practice (25–27).[5] Indeed, among the various empirical dose schedules used in patients, the intermittent single high dose seems to be preferred because of the long plasma half-life of AM and DR in man (26,28,29). However, controversial reports exist on general toxic effects in man when these compounds are given in widely or closely spaced schedules (26,30–32).

Considering the desirability of applying pharmacologic data when devising dosage schedules and the dependence of drug activity and toxicity on tissue distribution, more intensive experimental and clinical pharmacokinetic studies of AM and DR in different treatment schedules may be useful.

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Evaluation of VP-16-213 in Malignant Lymphoma and Melanoma[1,2,3]

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SUMMARY

Phase II studies utilizing VP-16-213 in the treatment of 56 patients with malignant lymphoma and 29 patients with malignant melanoma were carried out by the Southwest Oncology Group. All patients had received extensive prior therapy. The initial dose of VP-16-213 administered was 45 mg/m² by iv infusion over 30–60 minutes on Days 1–5 every 3 weeks but, because of severe myelosuppression in the lymphoma group, the dose was subsequently reduced to 35 mg/m². Only three partial regressions lasting 6, 2, and 1+ months were noted in 17 patients with Hodgkin's disease. No favorable responses were noted in 35 patients with non-Hodgkin's lymphoma including 16 with the diffuse histiocytic type. No responses were noted in patients with melanoma. The major toxic effect was myelosuppression. VP-16-213 appears to lack significant effectiveness in these previously treated diseases.

[Cancer Treat Rep 62:801–803, 1978]

VP-16-213 is one of several semisynthetic podophyllotoxin derivatives reported to have antitumor activity in experimental animal tumors (1,2) and in man (3–9). It is a mitotic inhibitor which results in metaphase arrest as well as inhibition of cells entering mitosis (2,9).

The reports of Nissen et al (3) and the European Organization for Research on Treatment of Cancer (4) suggesting significant activity of VP-16-213 in malignant lymphoma and activity in malignant melanoma prompted the initiation of these phase II studies in May of 1974.

METHODS AND MATERIALS

Patients with advanced histologically proven malignant lymphomas and malignant melanomas with measurable disease were eligible. All patients had received extensive prior chemotherapy, including vinca alkaloids in all lymphoma patients, and 60% of the lymphoma patients had received radiotherapy. Other criteria for eligibility included the following: written informed consent by the patient; no prior therapy with podophyllotoxins; an absolute granulocyte count $> 2000/\mu l$ and platelet count $> 100,000/\mu l$; a BUN < 30 mg/100 ml or serum creatinine < 2 mg/100 ml and total serum bilirubin < 2 mg/100 ml unless caused by the tumor; and no chemotherapy in the previous 21 days.

Pretreatment studies included a cbc count, blood chemistries, and chest and other roentgenograms as clinically indicated. Blood cell counts were obtained weekly, while blood chemistries and roentgenograms were obtained every 3 weeks. Tumor measurements were made every 3 weeks.

The initial dose of VP-16-213 was 45 mg/m² by iv infusion over 15–30 minutes on Days 1–5 every 3

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[3] VP-16-213 was supplied by the Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI, Bethesda, Maryland.

[4] This study was performed by the Southwest Oncology Group (SWOG). The following investigators participated in the study: *J. W. Cecil* (University of Utah Medical Center, Salt Lake City); *J. M. Quagliana* (Southern Nevada Memorial Hospital, Las Vegas); *C. A. Coltman* (Wilford Hall Medical Center, Lackland AFB, Tex); *M. Al-Sarraf* (Darling Memorial Center, Highland Park, Mich); *T. Thigpen* (University of Mississippi Medical Center, Jackson); and *C. W. Groppe, Jr* (Cleveland Clinic, Ohio).

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TABLE 1.—Schedule for dose adjustment

Absolute granulocyte count (cells/mm³)		Platelet count (cells/mm³)	Dose adjustment
>2000	and	>100,000	Increase 1 dose level
1000–1999	and/or	75,000–99,999	No change
500–999	and/or	50,000–74,999	Decrease 1 dose level
<500	and/or	<50,000	Decrease 2 dose levels

weeks as tolerated. Subsequent courses were repeated at 3-week intervals if the nadir of the wbc and platelet counts had been reached and evidence for recovery was present. Utilizing dose levels of 20, 30, 45, 60, and 75 mg/m² on Days 1–5, subsequent courses of drug were adjusted according to the degree of maximal myelosuppression according to the schedule in table 1. Two courses of treatment associated with myelosuppression were considered necessary for an adequate trial.

The response to therapy was classified as complete regression (CR), partial regression (PR), or increasing disease. Standard criteria were used with a PR indicating a reduction in tumor size by >50%.

RESULTS

Tumor Response

Fifty-six eligible patients with malignant lymphoma from 18 institutions and 30 eligible patients with malignant melanoma from 14 institutions were entered into these studies. Four patients were considered inevaluable due to protocol violation, insufficient data from the investigator, or death prior to drug administration.

Three PRs were noted in 17 evaluable patients with Hodgkin's disease. The durations of response were 6½, 2, and 1+ months with therapy being discontinued in the latter patient because of possible hepatotoxicity. No regressions were observed in 35 evaluable patients with non-Hodgkin's lymphoma including 16 with the diffuse histiocytic type. The remaining 19 patients were classified as having both nodular and diffuse lymphocytic or mixed non-Hodgkin's lymphoma.

All of the 29 evaluable patients with malignant melanomas treated with VP-16-213 were unresponsive.

Toxicity

Myelosuppression was the major toxic effect observed. In the lymphoma group, the incidence of severe and life-threatening neutropenia and thrombocytopenia prompted a reduction in the initial dose level to 35 mg/m². Noteworthy is the greater incidence of toxic effects in the lymphoma patients, who for the most part had received more extensive prior therapy than the melanoma patients. Table 2 demonstrates the distribution of myelosuppression in patients who were evaluable for myelotoxicity.

One patient died of an unknown cause within 12 hours after the first infusion of VP-16-213. Two patients developed transient jaundice and elevation of alkaline phosphatase that suggested the possibility of hepatotoxicity. In one patient, jaundice was noted 1 week after a 5-day course of VP-16-213 (45 mg/m²). In a second patient, who was having a PR, jaundice developed after his second course of VP-16-213 (60 mg/m²). A liver biopsy revealed chronic inflammation which was considered non-diagnostic but consistent with either viral hepatitis or drug toxicity. The occurrence of an acute myocardial infarction in a 27-year-old woman raised the question of drug-induced coronary artery-type cardiotoxicity (10). Although anorexia, nausea, vomiting, and alopecia were noted, they did not interfere with drug administration. Neurotoxicity was not observed.

TABLE 2.—Myelotoxic effects in patients receiving VP-16-213

	Absolute granulocyte count (cells/mm³)	Platelet count (cells/mm³)	No. of patients	
			Lymphoma	Melanoma
None	>2000	>100,000	10	18
Mild	1500–1999	75,000–99,999	11	6
Moderate	1000–1499	50,000–74,999	7	3
Severe	500–999	25,000–49,999	10	1
Life-threatening	<500	<25,000	11	1
Fatal			1	0

DISCUSSION

Clinical trials with VP-16-213 employed in a variety of schedules report significant antineoplastic activity in Hodgkin's disease and other lymphomas, particularly the histiocytic type (3–9). Cumulative overall response rates for other clinical trials are 23% in Hodgkin's disease, 22% in reticulum cell sarcoma, and 19% in lymphosarcoma (9). In contrast to these results, the negative results of this study led to the conclusion that VP-16-213 has minimal activity as a single agent in lymphoma patients having such extensive prior therapy. The degree of myelotoxicity observed in this study is more severe than that reported by other investigators utilizing similar doses and schedules and is attributed to prior antitumor therapy.

The results of this study demonstrate that VP-16-213 is ineffective in patients with malignant melanoma.

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Phase II Clinical Trial With Vindesine for Remission Induction in Acute Leukemia, Blastic Crisis of Chronic Myeloid Leukemia, Lymphosarcoma, and Hodgkin's Disease: Absence of Cross-Resistance With Vincristine[1]

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SUMMARY

Vindesine, an analog of vinblastine and vincristine, has been submitted to a phase II trial, the results of which are judged in terms of remission induction. A high proportion of remissions were obtained in acute lymphoid leukemia and blastic crisis of chronic myeloid leukemia, and a few responses have been registered in lymphosarcoma and Hodgkin's disease. A continuous 48-hour iv infusion may induce a remission where an iv push of the same dose has failed. The most remarkable characteristic of vindesine is the absence of cross-resistance with vincristine as documented in acute lymphoid leukemia.

[Cancer Treat Rep 62:805–809, 1978]

The two available vinca alkaloids studied during the last 20 years (1,2) are important clinical oncostatic agents: vinblastine is effective in Hodgkin's disease, vincristine is effective in acute lymphoid leukemia and lymphosarcoma, and both are effective in several solid tumors, especially in combinations (3).

The formulas of vinblastine and vincristine are very similar, differing only in one radical (fig 1). Although in both agents the most important mechanism of action is the inhibition of microtubule formation of the mitotic spindle resulting in arrest of cell division in metaphase (4), alterations in neurotubules (5), and changes in axoplasmic transport (6), their differences in clinical activities are well-demonstrated and generally accepted (3), and these differences parallel different biologic activities (7,8).

These concepts justify our interest in new vinca compounds. In 1965, we studied vinleurosine (9), a drug which shows only minor activity in lymphosarcoma. Over a 6-month period, we submitted vindesine (VDN), a new derivative of these agents, to phase I and II clinical trials.

MATERIALS AND METHODS

The chemical structure of VDN (10) is derived from vinblastine, as seen in figure 1, which also shows the structure of vincristine. Although the chemical structure of VDN is nearer to that of vinblastine, its oncostatic action in rodents is nearer to that of vincristine (8).

Comparative toxicity studies in mice and rats showed that VDN is more toxic than vinblastine but less toxic than vincristine (11). Toxic manifestations included anorexia, blood cytopenia, and atrophic intestinal mucosa. Chronic toxicity in dogs receiving weekly injections of different doses of VDN (0.04–0.16 mg/kg) revealed that it is also less toxic than vincristine in these experimental conditions (11).

Phase I clinical trials of VDN have been conducted by us (unpublished data) and by several other groups (12–14); pharmacokinetic studies have been carried out as well (15,16). These studies have shown that in man this agent induces side effects almost identical to those of vincristine. By comparing the conclusions of the above trials, we have deduced that the dose of 4 mg/m²/week is applicable to phase II trials.

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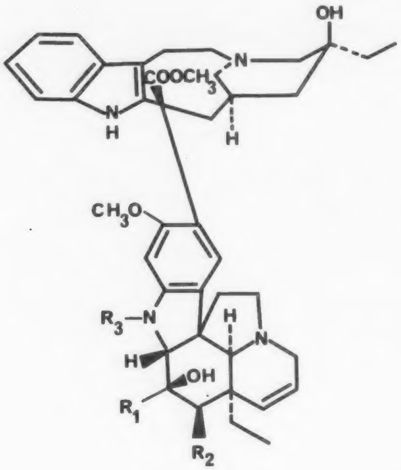
Chemical Name : 23-amino-4-deacetoxy-23-demethoxy-4-hydroxy-vincaleukoblastine, sulfuric acid salt

Molecular Formula : $C_{43}H_{55}N_5O_7 \cdot H_2SO_4$

Molecular Weight : 851.9

General Structural Formula of the Vinca Alkaloids



VINDESINE	VINBLASTINE	VINCRISTINE
R_1 = $CONH_2$	R_2 = $COOCH_3$	R_1 = $COOCH_3$
R_2 = OH	R_2 = $OCOCH_3$	R_2 = $OCOCH_3$
R_3 = CH_3	R_3 = CH_3	R_3 = CHO

FIGURE 1.—Developed formula of VDN compared to those of vinblastine and vincristine.

The present study followed the methodologic and ethical rules of all European Organization for Research on Treatment of Cancer phase II trials (17). It is evident that these guidelines tend to underestimate the effect of the compound, since most patients have been previously treated with available cancer therapy. The question arises as to whether some tumors will respond enough to justify further trials. Therefore, the results concerning the response rate are considered with caution since the patients have all received previous therapy.

The details concerning the patients, their diseases classified according to the World Health Organization (WHO) categorization (18), and their ages and sex are given in table 1. It is important to note that these patients are not in the initial phase of the disease but are in relapse, and are resistant to all previously available chemotherapeutic agents, except for patients who were resistant from the initial phase and patients with chronic myeloid leukemia in blastic crisis.

The efficacy in terms of complete remission (CR), partial remission (PR) (> 50% response), or failure and the toxicity were recorded.

VDN (supplied by Lilly-France) was given at a dose of 2 mg/m^2/day iv × 2 days (one cycle). In 13 patients, the dose was given by continuous perfusion over 48 hours. No other oncostatic agents were given along with VDN. There were 1-week intervals between the 2-day cycles. The total doses varied from 4.8 to 43.2 mg. The number of cycles and the total doses given were determined by the results obtained. VDN treatment was interrupted (*a*) when a CR was obtained (in order to administer maintenance therapy according to the protocol presently applied to each disease), (*b*) when a PR (> 50% response) was not enhanced by a new cycle, or (*c*) when a failure was observed 1 week after the second cycle.

A CR is defined as the disappearance of all perceptible manifestations, eg, clinical manifestations, blood and bone marrow leukemic cells, and

TABLE 1.—Patient characteristics

Disease	Total No. of patients	Patient age (yrs)/No. of patients	Patient sex/No. of patients	Disease type (WHO classification, ref 18)	No. of cases
Acute lymphoid leukemia	15	<15/8	F/8	Prolymphoblastic	1
		15–40/5	M/7	Macrolymphoblastic	2
		>40/2		Microlymphoblastic	0
				Prolymphocytic	5
				Immunoblastic	1
				Unclassified	6
Lymphosarcoma (stages III, IV. and leukemic)	13	<15/1	F/4	Prolymphocytic	6
		15–40/6	M/9	Lymphoblastic	2
		>40/6		Immunoblastic	3
				Unclassified	2
Hodgkin's disease (stage IV)	5	36–40	F/1		
			M/4		
Acute myeloid leukemia	3	12, 30, and 39	F/2	Myeloid	1
			M/1	Myelo-monocytoid	2
Blastic crisis of chronic myeloid leukemia	6	23–72	F/4	Myeloblastic-like cells	4
			M/2	Lymphoblastic-like cells	2

blood and bone marrow cytopenia in the case of acute lymphoid leukemia.

RESULTS AND DISCUSSION

Drug Tolerance

Among the toxic effects observed (table 2), the most common were neutropenia and thrombocytopenia (*see* definition in table 2), which were sometimes present and severe before VDN therapy, but increased after its use. Thrombocytopenia (< 10,000 cells/mm^3) was intense in three cases and may have been the cause of death in these patients by cerebro-meningeal hemorrhage. Neurologic manifestations were rare (table 2).

Oncostatic Effects in Terms of Remission Induction

The oncostatic effects are summarized in table 3 and the details of beneficial results are given in table 4. VDN is remarkably efficient in acute lymphoid leukemia, inducing six CRs (normal myelogram) and one PR (< 50% blast cells persisting in myelogram) among 15 patients. In two patients, the remission was achieved using a continuous infusion after iv push failure. One CR was obtained after one cycle, four were obtained after two cycles, and one was obtained after three cycles. The role of VDN in the duration of the CRs cannot be determined since the occurrence of a CR was followed by maintenance therapy with a protocol applicable to the disease.

VDN was less active in lymphosarcoma (WHO nomenclature) (18), resulting in only two PRs (> 50% response) among 13 patients. It did not induce any CRs, but did induce two PRs in Hodgkin's disease.

No remissions were obtained in acute myeloid leukemia, but three CRs were obtained among six patients with chronic myeloid leukemia in blastic crisis, a stage of the disease in which the best combination does not usually produce CRs at a rate of > 30% (19). One CR was obtained after one cycle and two were obtained after two cycles.

These results are remarkable, since patients submitted to phase II trials are patients with advanced disease, generally resistant not only to all drugs (prednisone, vincristine, L-asparaginase, adriamycin, cytosine arabinoside, cyclophosphamide, 6-mercaptopurine, and methotrexate in the case of acute lymphoid leukemias), but to the combinations known to be most efficient in these diseases (3). Table 4 shows, in particular, that six remissions were obtained in 15 patients with acute lymphoid leukemia resistant to vincristine, most of them in relapse.

Hence, VDN can be considered a very useful drug because of its possible efficacy in inducing remissions in neoplasms, especially acute lymphoid leukemia, resistant to all previously available effective drugs including vincristine. It may also be useful in the immunoblastic types of acute lymphoid leukemia (20) and in immunoblastic lymphosarcoma (21), both of which are very resistant to therapy.

TABLE 2.—Toxic effects (42 cases)

Toxic effect	
Neutropenia,* 22 cases (<1000 cells/mm³)	2 patients between 500 and 1000 cells/mm³ 20 patients <500 cells/mm³
Thrombocytopenia,† 18 cases (<100,000 cells/mm³)	9 patients <100,000 cells/mm³ 1 patient <50,000 cells/mm³ 3 patients <20,000 cells/mm³ 5 patients between 50,000 and 25,000 before treatment and <10,000 cells/mm³ after treatment
Anemia,‡ 7 cases	Hemoglobin decreased between 2 and 4.3 g/100 ml
Neurologic manifestations	2 patients (paralytic ileum) 1 patient (hyporeflexia) 1 patient (facial paralysis of short duration)
Skin manifestations	1 patient (generalized rash during 2 days) 1 patient (erythematous pruriginous rash)
Vomiting	1 patient
Fever	4 patients (fever spike 24 or 48 hrs after VDN administration)
Phlebitis	2 patients (1 from iv direct bolus and 1 from iv continuous infusion)
Lethal toxicity	3 patients (meningeal hemorrhage)

*2 patients were unevaluable because they had < 500 cells/mm³ before treatment.

†4 patients were unevaluable because severe thrombocytopenia (< 10,000 cells/mm³) was present before treatment.

‡5 patients were unevaluable because severe anemia (< 9 g/100 ml) was present before and after treatment.

TABLE 3.—Remission induction from VDN

		Results		
Diagnosis	No. of cases	CR	PR (>50%)	Failure
Acute lymphoid leukemia	15	6	1	8
Lymphosarcoma	13		2	11
Hodgkin's disease	5		2	3
Acute myeloid leukemia	3			3
Chronic myeloid leukemia, blastic crisis	6	3		3
Totals	42	9	5	28

TABLE 4.—Details of beneficial results in acute lymphoid leukemia (CRs)

Patient No.	Diagnosis	Perceptible phase	Last treatment (to which the disease is resistant)	Bone marrow leukemic cells (before and after each cycle of VDN)	Dose to achieve remission
1	Acute lymphoid leukemia, prolymphocytic	4th	Prednisone + vincristine + L-asparaginase	48%→16%→4%→1%	2 cycles; 2 mg, 2 days iv direct bolus
2	Acute lymphoid leukemia, immunoblastic	1st	Prednisone + vincristine + L-asparaginase	69%→2%	1 cycle; 2.4 mg, 2 days iv direct bolus
3	Acute lymphoid leukemia, prolymphocytic	2nd	Prednisone + vincristine + L-asparaginase	48%→48%→3%	2 cycles; 3.6 mg, 2 days iv bolus 1 cycle; 3.6 mg, 2 days iv continuous
4	Acute lymphoid leukemia, prolymphocytic	4th	Vincristine	9%→16%→5%→2%	2 cycles; 1.4 mg, 2 days iv bolus and 1.4 mg, 2 days iv continuous
5	Acute lymphoid leukemia, macrolymphoblastic	2nd	Prednisone + vincristine + L-asparaginase + 6-mercaptopurine + methotrexate	49%→15%→1%	2 cycles; 3 mg, 2 days iv bolus
6	Acute lymphoid leukemia, unclassified	2nd	Prednisone + vincristine + cyclophosphamide + 6-mercaptopurine + methotrexate	20%→5%	2 cycles; 3 mg, 2 days iv bolus

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Peptichemio in Advanced Neuroblastoma[1,2]

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SUMMARY

Peptichemio (PTC) is a mixture of six synthetic peptides of *m*-L-phenylalanine mustard. It acts with both alkylating and antimetabolic effects, interfering with the synthesis of DNA, RNA, and proteins. PTC was administered iv to 18 previously untreated children with advanced neuroblastoma at a dose of 1–1.5 mg/kg/day for one to three cycles of 5–6 consecutive days each. Eleven of 12 patients (92%) experienced both objective and subjective improvement; complete remission was achieved in two of them. In spite of the high remission rate, the median duration of remission has been short (4 months) and the overall survival (median, 6 months) did not seem to be influenced by the use of PTC. The primary toxic effects were, in order of importance, bone marrow depression, phlebosclerosis, nausea and vomiting, and alopecia. Chronic use of PTC seems limited by two major factors: profound long-lasting thrombocytopenia and severe phlebosclerosis.

[Cancer Treat Rep 62:811–817, 1978]

Peptichemio (PTC) is the trade name for an antitumor drug made up of six synthetic peptides of *m*-L-phenylalanine mustard (1,2) (fig 1). Its mechanism of action is based upon alkylating activity by the dichlorodiethyl-amino group and antimetabolic effect due to the peptidic moiety. This results in interference with the synthesis of DNA, RNA, and proteins (3–5).

PTC affects the cell cycle of the human heteroploid cells in G_1, S, and G_2 phases (6). The drug has activity against both the ascitic and solid forms of several transplanted animal tumors, ie, L1210 leukemia, Yoshida ascites sarcoma, Sarcoma 180, and Adenocarcinoma 755 (3). An oncolytic effect has been recently demonstrated in human neuroblastoma hetero-transplanted in nude mice (7). A good therapeutic index was obtained in preclinical toxicity studies performed in rodents and dogs (3). Cultured human and rat lymphocytes undergoing blastogenesis were only slightly affected by PTC (8–11). The drug does affect human chromosomes in vitro; it produces chromatid breaks, fragmentations, complex rearrangements, and tetraploidy (12), and may be mutagenic although studies of this property have not been published.

Since 1970, a number of clinical trials in adult patients with a wide range of tumors have been under way in Italy and other European countries. Pulmonary, gynecologic, urologic, and head and neck carcinomas were found to be responsive to PTC (4,12–14).

Among lymphoproliferative diseases, antitumor effects were obtained in patients with non-Hodgkin's lymphoma, multiple myeloma, and chronic leukemia (15–20). PTC has also been reported to be effective when directly injected into the pleural or peritoneal cavities of patients with metastatic effusions secondary to breast, lung, and ovarian carcinomas (21).

Finally, several authors have described an analgesic effect of the drug, suggesting that it could be considered in the management of tumor-related

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[2] Presented in part at the 67th Annual Meeting of the American Association for Cancer Research, Toronto, Canada, May 4–8, 1976.

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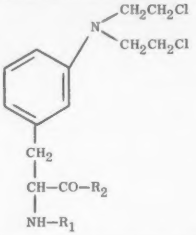
PARENT MOLECULE	R_1	R_2
	H-	-(NO_2)Arg. Nval
	Ser. pFPhe-	-OH
	Pro-	-pFPhe
	pFPhe-	-Asn
	m-SL. Arg. Lys-	-His
	pFPhe. Gly-	-Nval

FIGURE 1.—Structure of PTC.

intractable pain (16,19,20). These studies indicated that in man the toxic effects of the drug are relatively few at pharmacologic doses. In order of importance, these are bone marrow depression, phlebosclerosis, allergic reactions, nausea and vomiting, and alopecia (22).

The first use of PTC in pediatric cancers was by Massimo et al (11), who reported its effects in 33 children with solid tumors and in seven with acute leukemia. Tolerance to the drug was acceptable; toxic effects were limited to reversible leukopenia, thrombocytopenia, and alopecia. Three patients with neuroblastoma were in the group in which tumor responses were recorded. In a study by Madon et al (23), ten children with solid tumors resistant to conventional chemotherapy responded to PTC, and this group included two cases of generalized neuroblastoma. Both authors emphasized the beneficial effects of PTC on bone pain and the general status of patients with widely disseminated tumors. The aim of the present study was to further evaluate the efficacy of this drug in children with advanced neuroblastoma.

MATERIALS AND METHODS

Patients

Between January 1973 and April 1976, 18 children with widespread neuroblastoma (15 previously untreated and three with previous minimal chemotherapy) were entered in this study and received PTC as the initial drug. At the end of the trial, they were started on a sequential combination of vincristine and cyclophosphamide. Diagnosis was made by biopsy in ten patients; in the remaining eight, it was made on the basis of clinical, radiologic, and biochemical data.

Drug Administration

The drug is supplied in 0.5-ml vials for iv use; it contains 40 mg of active substance diluted in a nonaqueous solvent and is unstable in the presence of light or heat. The calculated daily dose is diluted in 100 ml of 5% dextrose in water and is administered over a 1/2-hour period. This is followed by an equal amount of 5% dextrose in water to minimize the risk of phlebitis. A single course of PTC consists of 1–1.5 mg/kg/day × 5–6 consecutive days. Unless progression of disease occurs, a second course is given 7–14 days later. The rest interval may be prolonged depending upon recovery from hematologic toxicity. In patients with a good response, a third course can be administered.

Methods of Observation

Twenty-four-hour urinary excretion of vanillylmandelic acid (VMA) was measured before starting therapy and, when possible, after each course. The values were compared with those from disease-free age-matched children.[5] VMA was determined by a modification of the method of Pisano et al (24). During therapy, patients were examined daily for signs of toxicity and evidence of therapeutic responses. Hemoglobin concentration and wbc and platelet counts were determined twice weekly or more frequently when necessary. The hematologic toxicity ratings are shown in table 1. Serum urea nitrogen, uric acid, glutamic oxaloacetic transaminase, total proteins, and erythrocyte sedimentation rate were determined at intervals of 1–2 weeks. A study of iliac crest marrow aspirate or biopsy was performed after treatment in patients with previously detected bone marrow invasion. Serial chest

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[5] Lamedica G. Unpublished data.

TABLE 1.—Evaluation of PTC in 12 children with advanced neuroblastoma

Patient No.	Sex/age	Site of primary/ type of surgery	Primary symptoms and signs of disease*	PTC therapy†	VMA‡	Response and clinical course*	Response category/ survival (mos)	Toxicity§
1	F/11 mos	Unknown/ biopsy	Vomiting, right jaw metastases	1.5/2	31.5–20	No response	0–0/6	A
2	F/2$^{1}/_{2}$ yrs	Left adrenal/ none	Bone pain, vomiting, AM, BM+, IVP+, MBL, OE&P	1.5/3	17–6.7	CR after 2nd course, relapse during 3rd course	0–C/2	A, L–3
3	M/6$^{1}/_{2}$ yrs	Left hemithorax/ biopsy	Chest pain, invasion of left pleura and diaphragm	1.5/3	12–2.1	<50% tumor regression for 12 mos, relapse with metastasis in right lung	I–A/18	A
4	F/3 yrs	Unknown/ none	Bone pain, BM+, MBL, OE&P, blindness, hepatomegaly	1/2	127–31.5	Recovery of sight, complete BM remission for 2 mos	I–A/8	A
5	M/7 yrs	Retroperitoneum/ biopsy	Bone pain, AM, BM+, left pleural effusion	1/2	37–58	Partial BM remission, normal chest roentgenogram for 5 mos	I–A/6	A, L–2, T–3
6	F/4 yrs	Right adrenal/ biopsy	AM, BM+, IVP+, metastatic adenopathies (neck)	1.2/2	ND–5.2	AM reduced and partial BM remission for 5 mos	I–A/12	A
7	M/1$^{1}/_{2}$ yrs	Left adrenal/ none	BM+, IVP+, skull lumps	1.5/3	13–4	CR for 6 mos, then new MBL	I–B/13	A, SR, L–2, T–2
8	M/5 yrs	Left adrenal/ biopsy	Bone pain, AM, BM+	1.5/2	60–5.7	AM reduced and partial BM remission for 4 mos, then new MBL	I–B/6	A
9	F/3 yrs	Right adrenal/ biopsy	Right pleural effusion, IVP+	1.5/2	69–1.17	CR for 4 mos, then MBL	I–B/4$^{1}/_{2}$	A, L–1, T–1
10	M/4 yrs	Right adrenal/ none	Bone pain, AM, BM+, MBL, IVP+, liver metastases	1/3	42–2.55	Normal IVP, complete BM remission and AM greatly reduced for 2 mos	I–B/6	A, L–3, T–3
11	M/5$^{1}/_{2}$ yrs	Left adrenal/ biopsy	Bone pain, BM+, MBL, metastatic adenopathies	1.5/3	11.5–5.3	Clinical CR and partial BM remission for 3 mos, then new MBL	I–B/4	A, L–3, T–1
12	F/2 mos	Left adrenal/ residual tumor excision after PTC	Respiratory distress, AM, skin nodules	1.5/1	19.5–0.45	Normal skin and AM greatly reduced after PTC, presently alive and well	I–C/32+	None

*AM = abdominal mass; BM = bone marrow; BM+ = bone marrow invasion; IVP = iv pyelogram; IVP+ = suprarenal mass shown by IVP; MBL = multiple bone lesions; OE&P = orbital ecchymoses and proptosis; CR = complete remission.

†(mg/kg/day)/No. of cycles.

‡Urinary excretion (mg/24 hrs) before and after PTC. Normal values are underlined. ND = not determined.

§A = alopecia; SR = skin rash; L = leukopenia; T = thrombocytopenia. Nos. refer to degree of toxicity: 1 = mild; 2 = moderate; 3 = severe (*see* Toxicity section for definitions).

and bone roentgenograms were made when tumors were discovered in the initial workup. Other studies were done when indicated.

Criteria of Evaluation

Failure to respond to PTC was defined as either progression of disease during or immediately after the first course, or lack of any improvement after the second course. Favorable responses were evaluated according to Karnofsky's response categories (25). However, the duration of responses has been determined with some limitations, since PTC was discontinued from 1 to 2 months after patients achieved a response.

RESULTS

Of the 18 children entered in this study, six were inevaluable because of early death (one patient), inadequate dosage of drug (two patients), and inadequate followup (three patients). The remaining 12 patients received sufficient drug according to the protocol and were considered evaluable. None of the children had received previous treatment, except for a few days of corticosteroids in patients 2 and 10 and one dose of vincristine in patient 6. No patient had radiation therapy during the trial. The data concerning the evaluable patients are summarized in table 1.

Antitumor Responses

Antitumor responses were observed in 11 of 12 patients (92%). In patient 1, the tumor did not grow after the first course but did during the second course. Patient 2 had objective regression of all measurable lesions except for elevated VMA excretion, and relapsed during administration of the third course of PTC.

Four patients experienced subjective improvement and partial tumor regression lasting >1 month, with remission durations of 2, 5, 5, and 12 months. Six patients exhibited major tumor regression with remissions lasting 2, 3, 4, 4, 6, and 15+ months respectively. In patients 7 and 9, complete remission was achieved.

VMA excretion was measured in 11 of 12 patients before starting treatment. It decreased by $\geq 50\%$ in ten patients following PTC therapy; however, only five of these children reached normal values.

All patients, except for patient 1, had subjective improvement, which usually occurred within the first few days of treatment. This manifested as improved appetite and activity, sense of well-being, and disappearance of bone pain. A variety of objective responses occurred, since different sites of tumor involvement were present when therapy was started. Changes in bone lesions were not included in this analysis which covers a relatively short time period. Fever, a constant feature at diagnosis, disappeared in 11 of 12 children in 1–3 days. Patients 6, 7, and 10 had abnormal IVPs for the presence of suprarenal masses, which became no more appreciable after PTC treatment.

In eight of eight patients with bone marrow involvement, remission (in terms of disappearance of extrinsic cells) was documented by needle aspiration; however, in two of three patients (Nos. 2 and 10) who had marrow biopsy by Jamshidi needle (26) done at the same site of aspiration, neoplastic cells in clusters were found. Orbital ecchymoses and proptosis present at diagnosis in patients 2 and 4 disappeared dramatically during the first PTC course; however, these findings recurred in patient 2 during the third course. Patient 4 was blind because of a possible involvement of her optic nerves. Following treatment, she recovered sight for a 2-month period.

Patient 9, who presented with an abdominal mass and right pleural effusion, underwent complete remission which lasted for a 4-month period (fig 2). Patient 12 represents a dramatic example of the activity of PTC in large neuroblastoma masses in young infants; this 2-month-old female infant presented with numerous skin nodules and an enormous abdominal mass, causing severe respiratory distress. During the administration of a single course of PTC, the skin cleared completely; in the following week the abdominal mass became barely palpable. One month later, laparotomy was performed and a 2 × 2-cm mass confined to the left adrenal was excised; no other evidence of tumor was found. No further therapy was given and she is alive and free of detectable disease 32 months after diagnosis.

Toxicity

The most important side effect is bone marrow depression. Leukopenia was seen in six patients (mild [2500–3000 cells/mm^3] in one, moderate [1500–2499 cells/mm^3] in two, and severe [<1500 cells/mm^3] in three). Thrombocytopenia occurred in five patients (mild [75,000–99,000 cells/mm^3] in three and severe [$<50,000$ cells/mm^3] in two). The nadir of both leukopenia and thrombocytopenia usually occurred a few days after completion of the second PTC course. Bone marrow depression was most profound and prolonged in patients with previous total marrow replacement (patients 5, 6, 10, and 11).

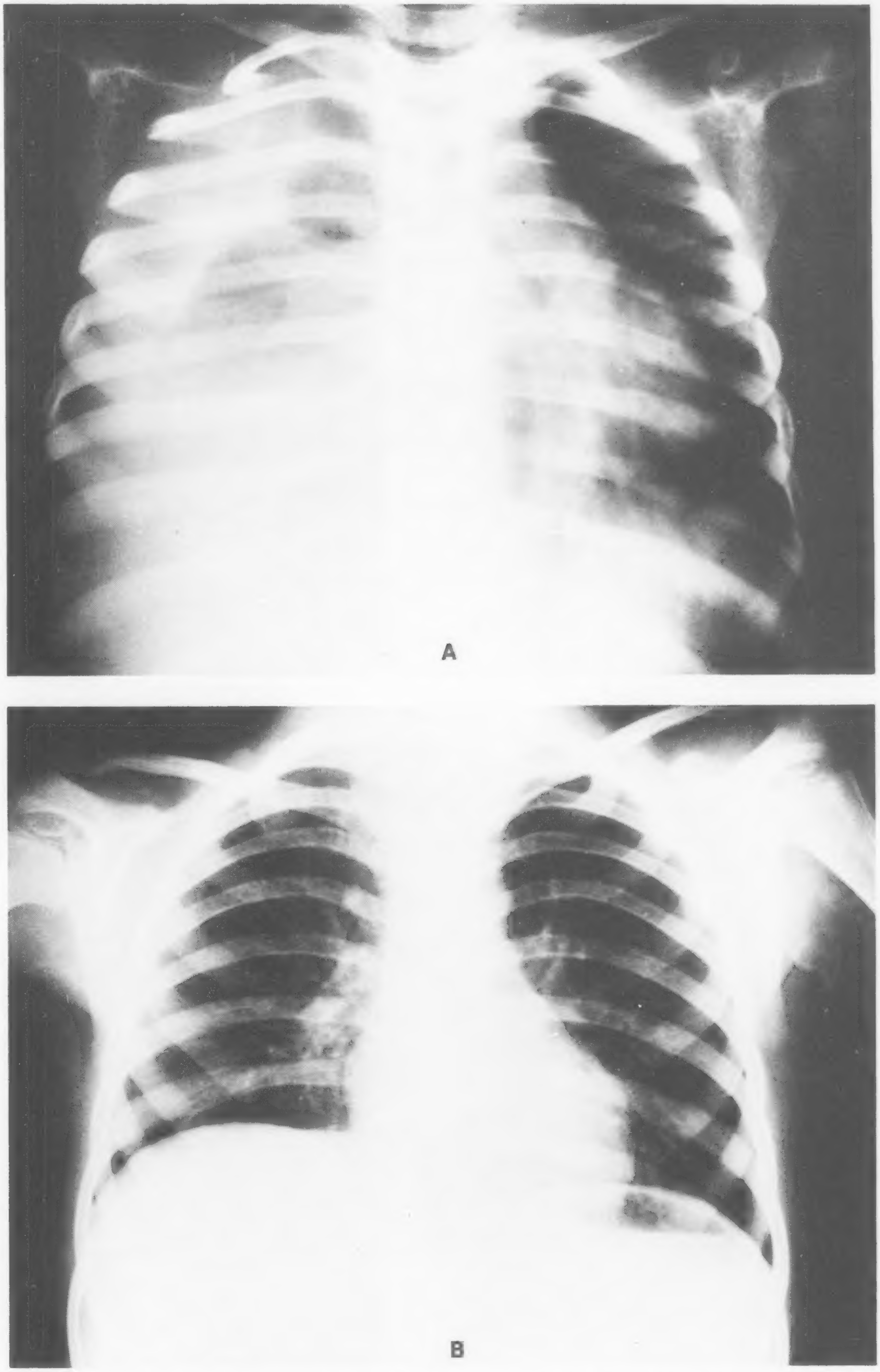

FIGURE 2.—Chest roentgenogram of patient 9 at diagnosis (A) and at completion of the second PTC course (B).

Mild to severe phlebosclerosis at the site of infusions occurred in children who received more than one course. Younger children were observed to be more affected than older children. Reversible alopecia occurred in all patients who received more than one cycle. A generalized skin rash was observed in one child (patient 7) who responded well to oral antihistaminics.

DISCUSSION

Our data indicate that PTC is an effective drug in previously untreated advanced neuroblastoma; only one of 12 adequately treated patients failed to show any response at all. The overall response rate of 92%, although of short duration, compares favorably with those obtained with other single antitumor agents known to be effective in advanced stages of the disease. Cyclophosphamide, the most active drug, gives percentages of response from 52% to 100% depending on the different schedules used (27–29), while daunorubicin (30), adriamycin (31), and vincristine (28,32) are effective in about 25% of the patients treated. However, it should be noted that the majority of patients in other series had had previous courses of antitumor agents, and it is known that the exposure to extensive prior chemotherapy usually implies a lesser degree of response and a higher frequency of toxic effects. Preliminary data from our Institute suggest that PTC is less effective when given to children with advanced neuroblastoma who had relapsed during other chemotherapeutic regimens. Each of four evaluable patients showed some kind of response to the drug, but the response lasted > 1 month in only one; in addition, myelosuppression appeared earlier and was relieved after a longer rest period.[6]

In spite of the fact that PTC has proved to be very active in patients treated for the first time, its influence on both duration of remission and survival time (4 and 6 months respectively) has been unremarkable in our patient series. Since in our protocol PTC is administered only in the induction phase, we cannot evaluate whether its use in a maintenance regimen would improve the results obtained so far.

Unfortunately, previous experiences have shown that the prolonged use of the drug (at least in the schedule adopted in the present protocol) was frequently followed by profound long-lasting thrombocytopenia and severe phlebosclerosis (11).[6] This, of course, indicates that PTC is not suitable for long-term treatment. Perhaps the exploration of different schedules of administration will better define the role of this agent in the primary treatment of childhood tumors.

ADDENDUM

Bone marrow biopsy has been a routine procedure at our Department of Hematology and Oncology since January 1975. So far, > 100 children with leukemias, solid tumors, and aplastic anemias have been examined at diagnosis and/or during treatment, for a total of 150 biopsies. In four cases of neuroblastoma with bone marrow initially invaded (two are mentioned in this paper), marrow remission documented by needle aspiration was not confirmed in the marrow biopsy performed at the same site by Jamshidi needle. This finding would suggest the utility of using bone marrow biopsy in children with neuroblastoma with bone marrow invasion in order to confirm remission.

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Peripheral Neuropathy as a Complication of *cis*-Dichlorodiammineplatinum(II) Treatment: A Case Report[1,2]

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SUMMARY

A 19-year-old woman with a diagnosis of osteosarcoma was initially treated with amputation of her right leg and adjuvant adriamycin. She developed pulmonary metastases 18 months following diagnosis. She was then given *cis*-dichlorodiammineplatinum(II) (DDP) at a dose of 100 mg/m^2 iv approximately every 4 weeks as the sole drug. Following the fifth dose of DDP, she complained of numbness and tingling in her hands and leg. A distal sensory loss extending to both elbows and her remaining knee was found on examination. Nerve conduction tests were compatible with peripheral neuropathy of the "glove and stocking" type. DDP was withheld and her sensory loss improved over the next 2 months, but became worse after another course of DDP was administered. The temporal relationship between the findings and the administration of DDP implicates this drug as the causative agent in the peripheral neuropathy.

[Cancer Treat Rep 62:819–821, 1978]

cis-Dichlorodiammineplatinum(II) (DDP) is an investigational antitumor agent which has been available for clinical trials since 1972 (1). DDP has been effective in a wide variety of tumors including osteosarcoma (2). The major toxic effects of DDP are renal, audiologic, hematologic, and gastrointestinal (2). In the current report, a 19-year-old woman with osteosarcoma treated with DDP developed a peripheral neuropathy.

CASE REPORT

This 19-year-old white woman presented in February 1975 at the age of 17 with pain and swelling of the right knee of 2 months' duration. Her past history was unremarkable. On physical examination, the right knee was firm with fullness, tenderness, and increased warmth over the medial femoral condyle. The range of motion was approximately 5°–130°. She had good dorsalis pedis pulses on both sides and patellar reflexes were normal. A detailed neurologic examination at that time was not done. A roentgenogram of the right knee showed a lytic lesion of the medial distal metaphysis of the right femur partly involving the epiphysis of the cortex. A bone scan showed increased uptake of the right distal femur. Her blood cell count, chemistry (including calcium, phosphorus, and serum alkaline phosphatase), and skeletal roentgenogram survey were all normal. On February 25, 1975, following an incisional biopsy, a tissue diagnosis of osteogenic sarcoma was made. A right high-thigh amputation was performed on February 28, 1975. She did well postoperatively and was treated with six courses of adriamycin to a total cumulative dose of 480 mg/m^2, this being completed in August 1975.

In June 1976, she developed a spontaneous left pneumothorax which required chest tube drainage for 4 days and then resolved. She did well until October 1976, at which time she developed a large pleural effusion. In November 1976, a chest roentgenogram showed a large radiopaque area occupying nearly the entire left chest. A thoracocentesis

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was done and 600 ml of serosanguineous fluid was removed. Malignant cells were noted on a cytocentrifuge specimen. Treatment was started with 50 mg/m^2 of DDP infused iv over 2 hours on 3 consecutive days. Each dose was preceded by a 2-hour period of intensive hydration (400 ml/m^2/hour) followed by a 24-hour infusion of 4000 ml/m^2 of fluid. Mannitol was used in the 6-hour forced diuresis following DDP infusion. Furosemide was not used. She had some nausea and vomiting and complained of tinnitus. An audiogram done 2 days after completion of this course showed a high-frequency loss in the right ear as well as suggestive sensorineural loss in the left. From December 1976 through April 1977, she received four additional 2-day courses of DDP, the first of which was 50 mg/m^2/dose while the others were 60 mg/m^2/dose. Following each infusion, she experienced nausea and vomiting. Audiograms showed further loss of high-frequency conduction. The BUN rose to 29 mg/100 ml and the creatinine to 1.3 mg/100 ml. One month following the fifth course of DDP, she began to complain of numbness and tingling in her hands and legs. On physical examination, she had hypoesthesia of both her hands and leg, more so in the hands. There was a distal sensory loss to both elbows and to the knee in the remaining leg. She had suggestive loss of proprioception and her handwriting was shaky. Her muscle tone and strength were normal and all deep tendon reflexes were elicited but reduced. Nerve conduction tests averaging several sensory stimuli showed no response in both the median and ulnar nerves of her left hand and the radial nerve of both hands. The motor conduction velocity was 59 m/second for the left median nerve and 56 m/second for the left ulnar nerve (normal range, 52.78 ± 5.15 m/second, elbow to wrist). Distal motor latencies were normal. This was felt to be compatible with a peripheral sensory neuropathy. Over the next 2 months, there was marked clinical improvement. Hence, in June 1977, she received her sixth 2-day course of DDP at 60 mg/m^2/dose. Approximately 1 month later she had deterioration of the neurologic condition marked by numbness in the hands and wrists. The patient emphasized the fact that there was a distinct relationship between the time of DDP administration and the reappearance of symptoms. Neurologic examination at this time revealed normal cranial nerves. Motor examination revealed good tone, strength, and development in the upper extremities. There was mild weakness of the left peroneus longus muscle. The only reflex that was obtained was the triceps reflex bilaterally. Other deep tendon reflexes were absent. The patient had a distal sensory loss in both hands and the remaining leg. There was suggestive loss of proprioception in both hands and none in the foot. Vibratory sensation was absent in the wrists and normal at the elbow and in the leg. The patient had a positive Tinel's sign when percussing the anterior interosseous nerve in the forearms and also on percussion of the common peroneal nerve on the left. A Tinel's sign could also be elicited when percussing the root of the sciatic nerve in the left thigh. This suggested incomplete damage to the peripheral nerve distal to the point of percussion. Averaged multiple sensory stimuli showed no response in the left median, ulnar, and radial nerve, and in the right ulnar nerve. Motor conduction velocity was 52 m/second for the left median nerve and 53 m/second for both ulnar nerves. No treatment was given other than to discontinue the DDP. At present, 3 months after her last dose of DDP, she still complains of some ringing in her ears and a mild tingling in her hands. Signs of her neuropathy have resolved. There is no sign of recurrence of the osteosarcoma.

DISCUSSION

Ototoxicity is a common complication of DDP therapy found in approximately 30% of the cases (3). It is manifested by tinnitus, or hearing loss limited to a frequency range above speech tones, or both, but a decrease of ability to hear normal conversation tones may occur. Auditory abnormalities have been described as the only toxic effects (4), and tinnitus has been described in the absence of hearing loss (5). Ototoxicity may be unilateral and more likely to occur with high-dose regimens (3). Histopathologic studies done in rhesus monkeys suggest that the hearing loss is due to loss of hair cells in the organ of Corti (6).

Although there is one poorly documented written report of peripheral neuropathy secondary to DDP (7), there have been several oral reports of neurotoxicity thought to be related to DDP. These have been collected by the NCI (Franco Muggia, personal communication); they include five seizures and 11 instances of peripheral neuropathy, both sensory and motor, occurring in the upper and lower extremities. Most of these patients had received vincristine, vinblastine, or hexamethylmelamine.

Since there are no data available on the mechanism of DDP toxicity, it is worthwhile to compare DDP toxicity with the toxicity of thallium, an element of the same series as platinum in the periodic table of chemical elements which also possesses antitumor activity (8).

Thallium salt poisoning has frequently been associated with peripheral neuropathy (9). In thallium toxicity, the neurologic symptoms are preceded by transient episodes of vomiting and/or diarrhea. Subsequently, painful paresthesias of the fingers and toes develop, followed shortly by weakness of the limb. Painful paresthesia ensues ≥ 1–5 days after ingestion. The legs and feet are usually more severely affected than the arms and hands. At the same time, numbness, especially of the fingers and toes, is noted with detectable loss of sensation to pin-prick and to touch. The involvement of vibration sense and sense of joint movement is less obvious. Both muscles and nerves can be painful to pressure. Motor weakness is also always distal in distribution and the lower limbs are more affected than the upper. Reflexes are characteristically perserved early in the course of the disease and are frequently brisk rather than reduced. In the case of chronic poisoning, all reflexes may be lost. All cranial nerves with the possible exceptions of I and VIII have been found affected. Experimentally, it has been shown that the conduction velocity of motor nerves is well maintained until the nerve becomes inexcitable (10). Pathologically, there are both degeneration of nerve fibers in peripheral nerves and chromatolysis of motor nerve cells (9). As for the mode of action of the thallium salt, it has been suggested that Tl^{++} ions act by replacing intracellular K^{+} ions as well as producing cellular damage by, as yet unknown, other mechanisms. Large doses of potassium chloride have been suggested as a possible antidote in acute thallium intoxication (9). DDP is a coordinated inorganic compound formed by a central atom of platinum surrounded by chlorine and ammonia atoms in the *cis* position in the horizontal plan (11). While its mechanism of action has not been fully elucidated, it appears to be that of an alkylating agent (8,12). The neurologic toxicity described in our case possesses many similarities to thallium neurotoxicity and is very likely due to DDP toxicity.

A much less likely possibility is that this patient's sensory neuropathy is a nonmetastatic complication of the malignant tumor. This has been documented in carcinoma of the bronchus (13) as well as in other tumors, namely Hodgkin's disease, seminoma, and malignant thymoma (14). To our knowledge, such an association has not been described in osteogenic sarcoma and the distinct temporal relationship between the administration of DDP and the recurrent appearance of symptoms speaks against this possibility.

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Inhibitory Effect of Hydroxynitrodihydrothymine on the Growth of Ehrlich Ascites Tumor Cells[1,2]

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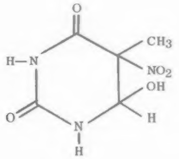
Certain nitroheterocyclic compounds, particularly nitrofuran and nitroimidazole derivatives with high electron affinity, have proved to be radiosensitizing as well as cytotoxic toward hypoxic tumor cells (1–7). These two important effects have increased the interest in this class of compounds for the treatment of cancer.

In this paper we present our findings on the inhibitory effect of hydroxynitrodihydrothymine (4-hydroxy-5-nitro-4,5-dihydrothymine; HNT) on the growth of tumor cells. The electron-affinic properties of this product have been previously described.[5]

MATERIALS AND METHODS

HNT was synthesized in our laboratory from thymine (8) purchased from Sigma Chemical Co (St. Louis, Mo). The drug, in the form of colorless crystals, has a molecular weight of 189.175 and a melting point of 182°C–183°C; it is soluble in water at a concentration of 1 mol/liter. In aqueous solution at pH 7.5 the compound shows ultraviolet maximum absorption at $\lambda = 310$ nm ($\log \epsilon = 3.37$). Electron affinity was measured by pulse radiolysis and was expressed as one electron reduction potential, $E^1_7 = -456$ mV.[5]



Ehrlich ascites tumor cells, Lettré variant, were maintained by weekly ip inoculation of 4×10^6 cells in NMRI male mice provided by the Cantacuzinc Institute in Bucharest.

Acute toxicity tests were carried out by ip administration of HNT at various concentrations, dissolved in 1 ml of saline (pH 3) or in isotonic 0.05 M phosphate saline buffers at pH 5 or 7.5. The toxic effect of each HNT dose was determined in groups of ten NMRI male mice. The observation period was 14 days and the LD50 was calculated by probit analysis.

The in vivo antitumor activity of HNT was tested in mice bearing Ehrlich ascites tumor cells injected ip (4×10^6 cells/mouse); the treatments started on the day after transplantation. All solutions were sterilized by passage through a millipore filter, HA 0.45 μm, before use. The experiments were conducted as follows:

a. Tests for the growth inhibitory effect of HNT as a function of pH were carried out by administering 3 × 1000 mg/kg/week of HNT dissolved in saline (pH 3.0) or isotonic phosphate buffers (pH 5 and 7.5). Seven days after transplantation, the animals were killed, the abdominal cavity was washed, the number of nucleated cells was counted in a Buerker chamber, and the mean values were calculated for each group of ten animals.

b. Determination of the dependence of the growth inhibitory effect of HNT on the size and number of doses and on the vehicle was performed by injecting HNT ip at doses between 500 and 2000 mg/kg, one to three doses per week; HNT was dissolved in either pHs, phosphate or tris buffer. The inhibitory effect was estimated in groups of ten mice using procedures described in *a*.

c. To determine the effect of repeated doses of HNT on tumor growth for a longer period of time, the drug was administered at doses of 1000 mg/kg for 4 weeks, using three doses per week. Ten animals were killed at the end of every week and

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[5] Wardman P. Personal communication.

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the number of tumor cells was estimated as described above.

d. The survival was recorded at 30 days in an experiment including four groups of 20 mice treated for 1, 2, 3, and 4 weeks, respectively (three doses per week, 1000 mg/kg of HNT).

During the experiments all mice received the same standard diet and water ad libitum.

The number of inflammatory cells in the abdominal cavity of untreated and HNT-treated mice was determined by washing the abdominal cavity of five mice in each group with saline and counting the nucleated cells in the washing fluid.

RESULTS

The acute toxicity of HNT in mice, expressed as the LD50, as well as the inhibitory effect on the growth of Ehrlich ascites tumor cells were dependent on pH (table 1). Saline solutions of HNT with a pH of about 3 caused the strongest toxic and inhibitory effects but they produced cell and tissue damage as well. On the other hand, HNT in neutral solution had very low toxicity and no inhibitory effects. For these reasons, we chose the pH 5 level for HNT solutions which we administered. Table 2 lists the comparative results for the growth inhibitory effect of HNT on tumor cells as a function of dose level and number of doses. At equivalent buffer concentration and pH the response was better when the drug was dissolved in tris than in phosphate buffer. A regimen of one to three doses per week of 500–1000 mg/kg of HNT in 0.5 ml of 0.05 M tris buffer at pH 5 seemed to be the most effective. Figure 1 shows that tumor cell growth was significantly inhibited within 4 weeks after transplantation when HNT was administered according to this schedule. Since the number of nucleated cells in the abdominal cavity of the treated animals (approximately 4 × 10^6 cells) was comparable to the number of inflammatory cells in untreated control animals (table 2), it appears that HNT treatments had completely suppressed tumor cell proliferation. It should also be mentioned that HNT administered ip induces an increase in the number of lymphoid and histiocytic cells in the abdominal cavity (table 2).

The proportion of mice surviving 30 days after tumor cell inoculation was increased by treatment with repeated doses of 1000 mg/kg of HNT (table 3).

TABLE 1.—Acute toxicity of HNT on NMRI mice and the growth inhibitory effect on Ehrlich ascites tumor cells as a function of pH

pH	HNT LD50 (mg/kg)	Growth inhibitory effect* (% inhibition vs control)
3	1800	97.5
5	2800	73.3
7.5	4000	None

*Three 1000–mg/kg injections per wk.

DISCUSSION

In regard to the mechanism of action of HNT, two factors must be considered: first, the toxic and inhibitory effects are dependent upon pH, and second, the possibility that the drug undergoes selective transformation in tumor cells with the production of toxic compound(s).

Our unpublished data revealed that, following neutralization, HNT was converted by ring opening into a salt of α-nitropropionic acid ureide, a product with low toxicity (LD50 = 4000 mg/kg) and no tumor growth inhibitory effects. The neutralization of HNT in vitro is an irreversible first-order reaction with a half-life of about 45 minutes.[6]

It has been postulated that the selective cytotoxic effect of nitrofurans and nitroimidazoles on hypoxic cells is a consequence of the enzymatic reduction of these compounds to amines via nitroso and hydroxylamine intermediates (9–11), and we feel it is reasonable to suggest a similar mechanism for HNT. In the greatly increased reductive conditions found in tumor cells, the nitro group of HNT could easily be reduced following the same reaction pathway. The occurrence of the hydroxylamine intermediate explains the toxic effect because these compounds react with proteins and DNA, producing inhibition of some biochemical pathways required for cellular viability (12–14). The difference in the inhibitory effect on tumor cell growth between HNT and its transformation product—the ureide of α-nitropropionic acid—indicates to us that the latter follows a different metabolic pathway.

The possibility of controlling the toxicity to the host organism and the growth inhibitory effect on tumor cells by the choice of an adequate pH, as well as the potential radiosensitizing effect against hypoxic tumor cells of HNT, could be exploited in the treatment of cancer.

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[6] Postescu ID, Mustea I, Pop R, et al. Chemical transformations of nitrohydroxydihydrothymine. Unpublished data.

TABLE 2.—Comparative growth inhibitory effect of HNT on Ehrlich ascites tumor cells as a function of the conditions of administration

Group	HNT dose (mg/kg)	HNT (doses/wk)	Vehicle*	No. of tumor cells/mouse ($\times 10^6$)	Statistical significance† (P)
1	—	—	—	210.98 ± 29.63	Control
2	—	—	Saline solution	156.87 ± 28.48	< 0.2
3	—	—	Isotonic phosphate buffer, 0.05 M	182.40 ± 20.44	< 0.3
4	1000	3	Isotonic phosphate buffer, 0.05 M	41.89 ± 8.06	< 0.001
5	—	—	Isotonic tris buffer, 0.05 M	150.70 ± 22.45	< 0.1
6	500	3	Isotonic tris buffer, 0.05 M	12.19 ± 4.24	< 0.001
7	1000	1	Isotonic tris buffer, 0.05 M	7.40 ± 1.58	< 0.001
8	1000	3	Isotonic tris buffer, 0.05 M	4.26 ± 0.63	< 0.001
9	1000	5	Isotonic tris buffer, 0.05 M	6.46 ± 1.12	< 0.001
10‡	2000	3	Isotonic tris buffer, 0.05 M	9.20 ± 0.68	< 0.001

The Nos. of inflammatory cells (lymphocytes and macrophages) in the abdominal cavity of untreated and HNT-treated control groups were $4.26 \pm 0.77 \times 10^6$ cells for the untreated control group, $4.44 \pm 0.61 \times 10^6$ at 7 days after treatment with 500 mg/kg of HNT 3 times a wk, $7.20 \pm 1.22 \times 10^6$ at 7 days after treatment with 1000 mg/kg of HNT 3 times a wk.

*In all experiments the pH of buffered solutions was 5; saline, isotonic phosphate, and isotonic tris buffer solutions were injected at 0.5 ml 3 times a wk.

†The statistical significance was calculated by the Student's t test.

‡40% mortality was observed.

TABLE 3.—30-day survival of mice receiving a standard inoculum of Ehrlich ascites tumor cells (4×10^6 cells) as a function of the No. of HNT doses*

No. of doses	Survivors (%)
0 (control)	45
3 (1 wk)	60
6 (2 wks)	80
9 (3 wks)	85
12 (4 wks)	90

*1000 mg/kg of HNT.

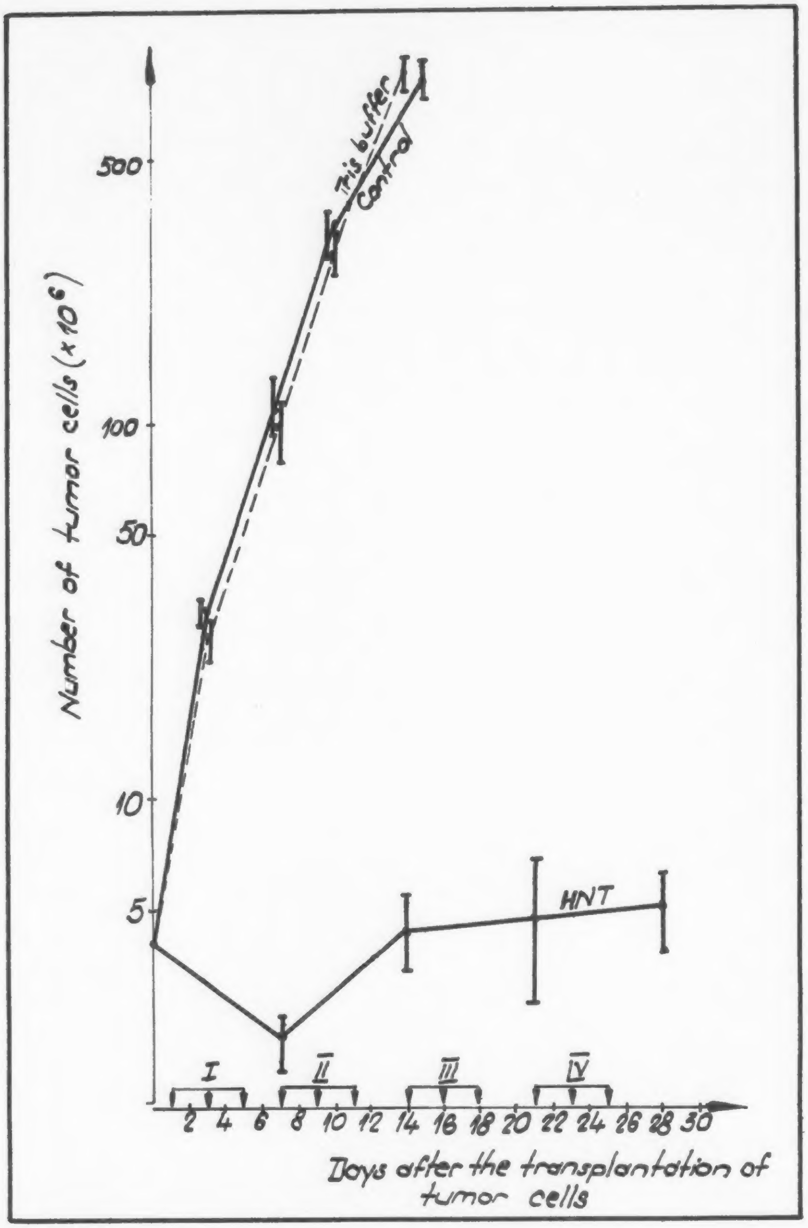

FIGURE 1.—Comparative effect of HNT on the growth of Ehrlich ascites tumor cells. HNT was given at 1000 mg/kg in 0.05 M isotonic tris buffer, pH 5. Roman numerals = No. of wks and arrows = days doses were given. Error bars on data points = SE.

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Actinomycin D in Childhood Acute Lymphocytic Leukemia[1,2]

Brief Reports and Preliminary Communications

Daniel M. Green,* Stephen E. Sallan, and Awtar Krishan[3,4]

SUMMARY

Eighteen evaluable children who relapsed with acute lymphocytic leukemia (ALL) were treated with intermittent, high-dose actinomycin D. Objective responses occurred in four of 11 children who had relapsed with chemotherapy which did not contain an anthracycline. The major toxic effects included thrombocytopenia and granulocytopenia. Minor toxic effects included nausea, vomiting, skin rash, and stomatitis. The onset of the maculopapular skin rash coincided with the platelet count nadir. These data suggested that actinomycin D is active in ALL.

[Cancer Treat Rep 62:829–831, 1978]

Actinomycin D, an antitumor antibiotic produced by *Streptomyces parvullus*, is highly active in a variety of experimental tumor systems (1) and several human solid tumors (2). The drug was reported to have "no effect on the course of acute leukemia in children" (3), although one study suggested that actinomycin D prolonged remission in childhood acute lymphocytic leukemia (ALL) (4); other studies in childhood ALL yielded equivocal results (5,6).

The clinical pharmacology of actinomycin D has recently been elucidated. Actinomycin D has a long tissue half-life (7) and a long plasma half-life (7,8), and is a cycle- and phase-non-specific agent (9). These results suggested that activity of actinomycin D in ALL might be demonstrated if the drug was administered on an intermittent dose schedule. We report here the results of a phase II trial of actinomycin D in childhood ALL.

PATIENTS AND METHODS

Twenty patients with ALL who failed conventional multiple-drug chemotherapy received an adequate trial of actinomycin D. An adequate trial of actinomycin D was defined as the administration of one dose and survival of the patient for at least 14 days following administration. As shown in table 1, most patients were having their second or third relapse. Patients 1 and 2 (of the group without adriamycin chemotherapy) in table 1 relapsed after elective cessation of therapy.

The patients and their parents were advised of the experimental nature of the proposed treatment, and informed written consent was obtained. All patients had relapse confirmed by bone marrow examination, and either had received no chemotherapy for 2 weeks or had progressive disease while receiving chemotherapy. Additional studies included cbc and platelet counts and liver function tests.

Actinomycin D, 2 mg/m², was administered as an iv bolus. Second and subsequent doses were administered every 14–21 days if continued treatment was consistent with optimal patient care. Bone marrow aspirations were obtained weekly between the first and second doses of actinomycin D and as indicated by response thereafter. Patient response was defined as complete (≤ 5% lymphoblast cells) or partial (≤ 25% lymphoblast cells) bone marrow remission (normocellular bone marrow), with no circulating lymphoblast cells and normal peripheral blood cell counts.

RESULTS

Twenty-one patients received actinomycin D. Of these, three were unevaluable: one who died of

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TABLE 1.—Patients in relapse from previous chemotherapy with or without adriamycin

Patient No.	No. of prior relapses	No. of courses	Response to actinomycin D (duration)	Subsequent clinical course*
			Without adriamycin	
1	1	1	PR	NED
2	1	3	PR	NED
3	3	5	CR (11 wks)	Failed prednisone-methotrexate; DOD
4	3	6	PR (11 wks)	Failed vincristine-prednisone; DOD
5	1	1	None	Failed vincristine-prednisone and L-asparaginase; DOD
6	2	1	None	Failed vincristine-prednisone; DOD
7	2	1	None	Failed vincristine-prednisone and L-asparaginase; DOD
8	2	2	None	CR with neocarzinostatin; DOD
9	2	1	None	DOD
10	2	2	None	DOD
11	2	3	None	PD
			With adriamycin	
1	No remission	1	None	DOD
2	2	1	None	DOD
3	2	3	None	DOD
4	2	2	None	Failed vincristine-prednisone, cyclophosphamide, and cytosine arabinoside; DOD
5	3	1	None	Died from actinomycin D-related sepsis
6	4	2	None	DOD
7	7	1	None	Re-induced with vincristine-prednisone; DOD

*NED = no evidence of disease; DOD = died of disease; and PD = progressive disease.

TABLE 2.—Hematologic parameters

	Prior to treatment	Nadir after 1st course	Nadir after subsequent courses
Wbc count (cells/mm^3)			
Median	1900	1000	1800
Range	(900–48,000)	(100–4100)	(100–3700)
Granulocyte count (cells/mm^3)			
Median	1116	54	414
Range	(0–3210)	(0–2870)	(30–1200)
Platelet count (× 10^3 cells/mm^3)			
Median	81	25	30
Range	(13–512)	(2–68)	(7–110)

leukemia 3 days after drug administration, one whose bone marrow went from 83% to 36% lymphoblast cells over 2 days without treatment, and one who received prednisone, 40 mg/m²/day × 4 days.

Of the 18 evaluable patients, 11 had relapsed with chemotherapy which did not include an anthracycline. Of the 11, one achieved a complete remission (CR) and three a partial remission (PR). The CR was achieved after one dose of actinomycin D, while the PRs were observed after one (one patient) or three (two patients) doses of actinomycin D. Seven of the patients in this group failed to enter remission with actinomycin D. One patient obtained a CR with neocarzinostatin while all others failed to respond to conventional reinduction programs (table 1). Seven additional patients either failed to enter a primary remission with multi-agent chemotherapy which included an anthracycline, or relapsed with combination chemotherapy which included an anthracycline. There were no responses to actinomycin D among these seven patients. One patient subsequently had re-induction with vincristine and prednisone while the remaining six patients had disease refractory to all treatment and died.

Toxicity

The major toxic effects were hematologic. All patients experienced profound thrombocytopenia following the initial dose of actinomycin D, generally 7 days after drug administration. Two patients were hospitalized for fever and granulocytopenia. One patient died from *Pseudomonas* sepsis. As shown in table 2, granulocyte count nadirs improved with subsequent drug administration, whereas platelet count nadirs remained unchanged. Mild-to-moderate nausea and vomiting occurred in all patients, lasting 6–18 hours after actinomycin D administration. Other toxic effects included stomatitis after ten (27%) of 37 courses, pruritic maculopapular rashes in five (14%) of 37 courses, and mild occipital radiation recall in a patient who had recently completed craniospinal irradiation. Significant abnormalities of liver function tests were observed in only one patient who had pre-existing antimetabolite-induced hepatitis.

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Chemotherapy of Gynecologic Cancer With Nitrosoureas: A Randomized Trial of CCNU and Methyl-CCNU in Cancers of the Cervix, Corpus, Vagina, and Vulva[1]

Brief Reports and Preliminary Communications

G. A. Omura,* H. M. Shingleton, W. T. Creasman, J. A. Blessing, and R. C. Boronow[2]

The nitrosoureas have a relatively broad spectrum of antitumor activity. Since they had not been extensively tested in gynecologic cancer (1) and since the relative activity of CCNU and methyl-CCNU was uncertain, a randomized trial was done. Our results in mustard-resistant cancer of the ovary have been reported separately (2).

MATERIALS AND METHODS

Women with biopsy-proven cancer of the genital tract whose disease was not amenable to further surgery or radiotherapy were eligible if there was at least one easily measurable mass, if the patient had recovered from toxic side effects of prior treatment, if she had an estimated survival of ≥ 3 months, and if written informed consent for protocol treatment had been given. Prior drug treatment was allowed, but not with nitrosoureas. Since most eligible patients would have had prior pelvic radiotherapy, the drug doses were selected in anticipation of limited tolerance for myelotoxic drugs. CCNU was given at a dose of 100 mg/m^2 or methyl-CCNU was given at a dose of 150 mg/m^2 every 6 weeks orally on an empty stomach, if the wbc count was $> 4000/\text{mm}^3$ and the platelet count was $> 150{,}000/\text{mm}^3$. Blood cell counts were to be obtained every 2 weeks. Random assignment of treatment was done by the closed-envelope technique. (CCNU and methyl-CCNU were supplied by the Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute.)

A complete regression (CR) was defined as the disappearance of all measurable tumor and relevant symptoms. A partial regression (PR) was defined as a > 50% reduction in total tumor area. Improvement was defined as between 25% and 50% reduction in total tumor area. Static disease was defined as a < 25% change over baseline measurements. Progression was defined as a > 25% increase in the total tumor area in one measurable lesion or the appearance of a new lesion. Any change in tumor measurement must have continued for ≥ 4 weeks to be significant.

Nine patients were ineligible (second primary, three patients; wrong primary, two patients; wrong cell type, one patient; leukopenia, one patient; and poor risk, two patients). Reasons for inevaluability included missing data (77 patients), chemotherapy violation (20 patients), clerical error (two patients), and inadequate pathology material (three patients).

RESULTS

Response

Results are shown in table 1. Of the 58 evaluable patients with squamous carcinoma of the cervix receiving CCNU, there was one CR lasting 54+

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[2] This study was performed by the Gynecologic Oncology Group (GOG). The writing committee included *G. A. Omura* and *H. M. Shingleton* (University of Alabama in Birmingham); *W. T. Creasman* (Duke University Medical Center, Durham, NC); *J. A. Blessing* (GOG Statistical Office, Roswell Park Memorial Institute, Buffalo, NY); and *R. C. Boronow* (University of Mississippi, Jackson).

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TABLE 1.—Nitrosoureas in gynecologic cancer

	No. of—					Response				
			No. of courses							
Diagnosis	Patients entered	Evaluable patients/ drug received	1	2	3+	CR	PR	Improvement	Static disease	Progression
Squamous carcinoma, cervix	196	58/CCNU	15	17	26	1	1	1	15	40
		62/methyl-CCNU	14	18	30	1	2	3	7	49
Adenocarcinoma, cervix	22	11/CCNU	5	2	4					11
		3/methyl-CCNU	1	1	1					3
Adeno-squamous carcinoma, cervix	13	5/CCNU	1	1	3					5
		4/methyl-CCNU	1	2	1					4
Endometrial adenocarcinoma	12	1/CCNU	1							1
		5/methyl-CCNU	2	2	1		2			3
Uterine sarcoma	20	6/CCNU	4		2	1				5
		6/methyl-CCNU	2	2	2					6
Squamous carcinoma, vagina	9	3/CCNU			3					3
		2/methyl-CCNU			2				1	1
Squamous carcinoma, vulva	12	4/CCNU		1	3					4
		3/methyl-CCNU		1	2					3
Totals	284	173								

months (pelvic recurrence) and one PR lasting 8 weeks (abdominal involvement). Of the 62 patients receiving methyl-CCNU, there was one CR of lung metastases (subsequently lost to followup) and two PRs lasting 8 weeks (liver) and 12 weeks (vaginal recurrence). Only six of the evaluable patients and none of the responders had had prior chemotherapy. The age range for all evaluable patients in this category was 21–79 years (median, 49) and their median survival from entry on the protocol was 5.4 months (PR, 14 months; improvement, 10 months; static disease, 5.5 months; and progression, 4.8 months).

The two PRs among six evaluable patients with endometrial adenocarcinoma lasted 8 weeks (pulmonary) and 11 weeks (pelvic). One patient with pelvic involvement by a leiomyosarcoma who had failed therapy with vincristine, actinomycin D, and cyclophosphamide showed complete disappearance of disease and was well 36 months later, having received 12 courses of CCNU. The patient with squamous carcinoma of the vagina rated as static disease received 17 courses and had stable lung metastases for 24 months.

Toxicity

There were 60 patients with squamous carcinoma of the cervix evaluable for hematologic toxicity (34 patients receiving CCNU and 26 receiving methyl-CCNU). Eight of 26 patients receiving methyl-CCNU and seven of 34 patients receiving CCNU had severe toxicity with a wbc count $< 2000/\text{mm}^3$ or a platelet count $< 50{,}000/\text{mm}^3$.

In each treatment arm there was one case of life-threatening thrombocytopenia (11,000 cells/mm^3 after two courses of methyl-CCNU and 20,000 cells/mm^3 after three courses of CCNU). Both patients had had prior pelvic radiation but no other chemotherapy. Nausea was common with both drugs but was not dose-limiting. Other side effects were not documented. The low rate of evaluability for toxicity resulted primarily from insufficient interim blood counts.

DISCUSSION

Response rates of $\geq 20\%$ for single agents such as cyclophosphamide, 5-fluorouracil, vincristine, adriamycin, and mitomycin C in cervical cancer have been tabulated (3) and improved results using drug combinations have been reported (4), especially for unirradiated or extrapelvic sites of disease. In the present study, the majority of patients had pelvic recurrences. Nevertheless, there were 27 patients with squamous carcinoma of the cervix who had pulmonary metastases, either alone or in conjunction with pelvic disease. Of these, there were only five who showed any response of their lung lesions.

It might be questioned whether the drug doses were adequate in this trial, but since 25% of the cervical cancer patients evaluable for toxicity experienced severe hematologic toxicity, it appears that these drug doses were adequate for this patient population. The achievement of only five responses (two CRs and three PRs) in 120 evaluable patients indicates that these doses of CCNU and methyl-CCNU have little activity in squamous cervical cancer. No advantage was apparent for either drug. There were too few evaluable cases in other types of gynecologic cancer to establish response rates, but the responses in endometrial carcinomas (two PRs among six patients) and sarcomas (one CR among 12 patients) are of interest.

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Phase II Study of Triazinate and Pyrazofurin in Patients With Advanced Breast Cancer Previously Exposed to Cytotoxic Chemotherapy [1,2]

Brief Reports and Preliminary Communications

W. C. Nichols,* L. K. Kvols, J. N. Ingle, J. H. Edmonson, D. L. Ahmann,
J. Rubin, and M. J. O'Connell[3]

SUMMARY

The purpose of this phase II clinical trial was to evaluate the effectiveness of triazinate and pyrazofurin in the treatment of advanced breast cancer. Although an occasional patient experienced stability of disease, neither agent, as employed in this study, was able to produce an objective antitumor effect in this group of patients with previous chemotherapeutic exposure. At the dosages used in this study, neither agent can be recommended for further treatment of advanced breast cancer.

[Cancer Treat Rep 62:837–839, 1978]

Multiple-drug chemotherapy programs have induced objective responses in 45%–60% of patients with advanced breast cancer, as previously reported (1). There is still a need, however, to develop improved primary chemotherapy programs and to investigate new agents effective as secondary treatments following prior chemotherapy failures. Triazinate (TZT) and pyrazofurin (PRZF) each have shown some activity in breast cancer patients during phase I trials (2–4), yet neither agent has been adequately evaluated in patients with advanced breast cancer. This study assessed these two new agents utilizing a prospective randomized clinical trial in a group of breast cancer patients who had previously failed one or more combination-chemotherapy programs.

TZT (Baker's antifol) is a specific inhibitor of dihydrofolate reductase developed by B. R. Baker (5). Methotrexate, an agent found in numerous clinical trials to be effective in 20%–40% of patients with advanced breast cancer, inhibits this same enzyme system (6). TZT was accepted for early clinical trials on the basis of its ability to cure the ascites form of the Walker 256 rat carcinosarcoma and on its ability to enter the cerebrospinal fluid after iv injection in dogs. Phase I studies have identified dose-limiting toxicities of somnolence and dermatitis (2,3). Acceptable myelosuppression has occurred by using the drug in a 3-day course at 250 mg/m^2/day repeating courses every 3–4 weeks, except in patients with significant liver impairment in whom serum levels have been found to be markedly elevated (2). This same study included a clinical response in one of three patients with breast cancer. Decreased cerebrospinal fluid pleocytosis was also observed in a patient with breast cancer metastatic to the central nervous system during these earlier studies (3).

PRZF is a product of *Streptomyces candidus* fermentation. It inhibits DNA replication by interfering with orotidylic acid decarboxylase (4). PRZF has shown antitumor effect in a variety of experimental tumors including several DMBA-induced mammary carcinomas in rats (4). Previous human studies have reported toxicity to be primarily myelosuppression, stomatitis, and perianal dermatitis (7). Acceptable toxicity was noted using a dose of 240 mg/m^2 given once iv every 4 weeks (7).

MATERIALS AND METHODS

Thirty-four patients with histologically proven metastatic breast cancer were entered in the study and by random selection received either TZT or PRZF. Both primary treatment groups were comparable with respect to age, site of dominant disease, performance score, disease-free interval, and postmenopausal status (table 1). All patients had received prior cytotoxic combination chemotherapy.

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TABLE 1.—Patient data

	Drug	
	TZT	PRZF
No. of patients entered	17	17
Median age (yrs)	58	58
Range	38–76	43–74
Postmenopausal status (yrs)		
<1	2	0
1–5	2	4
5–10	5	5
>10	8	8
Disease-free interval (yrs)		
<1	4	4
1–5	9	9
>5	4	4
Dominant disease		
Visceral	12	10
Soft tissue	3	3
Osseous	2	4
Performance score		
0–1	9	10
2–3	8	7

Thirty of the 34 patients had failed more than one treatment program. The majority of prior chemotherapy courses consisted of combinations of cyclophosphamide, 5-fluorouracil, prednisone, adriamycin, melphalan, and VP-16-213. Two patients assigned to receive TZT during the study had experienced brief prior exposure to methotrexate. All patients had expected survivals of > 2 months and had measurable disease.

Each patient was evaluated with pre- and post-treatment assessments as previously described (1). A standard definition of objective response was utilized: a ≥ 50% decrease in the product of the perpendicular diameters of all indicator lesions without the appearance of new lesions. Progression of disease was defined as a ≥ 25% increase of previous lesions or the appearance of new lesions.

TZT was administered by iv infusion over 30 minutes on each of 3 consecutive days at a dose of 250 mg/m²/day repeated every 4 weeks unless progression was noted. PRZF was initially administered over 3 consecutive days at 80 mg/m²/day repeated every 4 weeks. This was altered after the first eight patients to a single iv infusion of the entire dose of 240 mg/m² at one time since a concurrent study using PRZF for the treatment of other tumors at this institution revealed less toxicity when the drug was given as a single iv infusion.[4] A 50% dose reduction of both drugs was used for patients with significant renal or liver disease (serum creatinine > 2 mg/dl, bilirubin > 2 mg/dl, or serum alkaline phosphatase over twice normal attributed to liver disease). The study design allowed for crossover to the alternate drug after progression on the primary therapy.

RESULTS

Of the 34 patients entered in the study, 17 received TZT and 17 received PRZF as primary treatment. Ten patients received TZT after progression on PRZF and five patients received PRZF after progression on TZT. There were no objective responses for either agent used as primary or as crossover treatment. Progressions were noted in ten of 17 patients (57%) in each treatment group during the first followup re-evaluation at 4 weeks. The best clinical result achieved with either drug was stability of previously progressive disease. Seven of 17 patients treated with TZT remained stable for a median time of 85 days (range, 39–255+) after initiation of treatment. Seven of 17 patients remained stable after initiation of treatment with PRZF for a median time of 57 days (range, 56–87). Overall, for the primary treatment groups, the median survival was 102 days (mean, 118) for TZT-treated patients and 112 days (mean, 130) for PRZF-treated patients. Five of the TZT and eight of the PRZF initially treated patients were still living at the time of this report.

Moderate to severe stomatitis occurred in 13 of 27 patients receiving TZT and in 11 of 22 receiving PRZF. Myelosuppression was generally mild with both drugs. The median leukocyte nadir was 3900/mm^3 (range, 2100–6800/mm^3) for TZT and 4300/mm^3 (range, 2200–6600/mm^3) for PRZF. The median platelet nadir was 190,000/mm^3 (range, 10,000–470,000/mm^3) for TZT and 171,000/mm^3 (range, 55,000–315,000/mm^3) for PRZF. There were no drug-related infectious complications or deaths; however, two patients with extensive hepatic disease received a dose of 250 mg/m² of TZT for 3 days and developed severe thrombocytopenia. One of these patients required hospitalization for gastrointestinal bleeding.

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1,2:5,6-Dianhydrogalactitol in Advanced Breast Cancer[1,2]

Brief Reports and Preliminary Communications

B. Hoogstraten, R. O'Bryan, and S. Jones[3,*]

Dianhydrogalactitol is the major conversion reaction product resulting from the treatment of dibromodulcitol with mild alkali or human serum (1). It inhibits synthesis of DNA, RNA, and protein. In a phase I study (2) of dianhydrogalactitol, a maximum tolerable dose for patients with prior chemotherapy was determined to be 30 mg/m²/day × 5 days. However, prior nitrosourea therapy resulted in more severe toxicity and a dose of 25 mg/m²/day × 5 days was recommended in such instances. Myelosuppression was the dose-limiting toxicity, especially in patients with liver function impairment. Nausea and vomiting were mild to moderate. Based on this phase I information the Southwest Oncology Group (SWOG) conducted this phase II trial of dianhydrogalactitol in patients with advanced breast cancer.

MATERIALS AND METHODS

Twenty-six patients with advanced breast cancer included in this study were treated at nine institutions. All had received prior treatment with drugs of known efficacy and were not eligible for protocols with higher priority. Only three had previously been treated with a nitrosourea. An expected minimum survival of 6 weeks was required and all patients had to have measurable disease. The nadirs of the blood cell counts resulting from prior chemotherapy had to be passed and patients had to have an initial granulocyte count of ≥ 2000/mm³ and a platelet count of 150,000/mm³.

Inadequate data made two patients unevaluable and one patient died on the third day of the first treatment course. Thus, 23 patients were fully evaluable.

TABLE 1.—Dose adjustments for dianhydrogalactitol

Absolute granulocyte nadir (cells/mm³)		Platelet nadir (cells/mm³)	Dose adjustment
>2000	and	>100,000	+20%
1000–1999	and/or	50,000–99,000	No change
500–999	and/or	25,000–49,999	–20%
<500	and/or	<25,000	–40%

Nineteen patients started receiving dianhydrogalactitol at doses of 30 or 25 mg/m² × 5 days by iv push, but four initially received a lower dose to offset anticipated severe toxicity. This was done at the discretion of the responsible investigator in patients who had demonstrated poor tolerance to chemotherapy.

Subsequent courses were administered at 4-week intervals as tolerated and were not repeated until the nadirs of the granulocyte and/or platelet counts had recovered to ≥ 1000 and 75,000/mm³ respectively. Blood counts were obtained biweekly. Subsequent courses of dianhydrogalactitol were adjusted in relation to the nadir blood counts as shown in table 1.

A > 50% reduction in the sum of the areas of all measurable lesions for ≥ 1 month with no appearance of new lesions was required in order to qualify for a partial response.

RESULTS

Thirteen of the 23 patients had progressive disease after receiving dianhydrogalactitol. Their median time in the study was 36 days (range, 24–61). Three of these patients died before a second course could be started, while the other ten received two courses. No change was noted in five patients and improvement other than a partial response was seen in four patients who were in the study for 91, 98, 118, and 154 days respectively. One patient has been in the study for > 9 months and has achieved a partial remission.

The doses used and the resulting myelotoxicity

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[3] This study was conducted by the Southwest Oncology Group. The following institutions participated in the study: University of Kansas Medical Center, Kansas City (*B. Hoogstraten*); Henry Ford Hospital, Detroit, Mich (*R. O'Bryan*); and University of Arizona Health Sciences Center, Tucson (*S. Jones*).

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TABLE 2.—Correlation of dose schedule and myelosuppression after the 1st course

Dose schedule (mg/m^2 × 5 days)	No. of patients	No. of patients with wbc count (cells/mm^3)—					No. of patients with platelet count (× 10^3 cells/mm^3)—				
		≥4000	3000–3999	2000–2999	1000–1999	<1000	≥100	75–99	50–74	25–49	<25
30	13	6	1	3	2	1	8	—	2	1	2
25	6	1	1	2	1	1	3	—	—	2	1
20	2	2	—	—	—	—	1	—	—	1	—
15	2	1	—	—	—	1	—	1	—	—	1

are shown in table 2. Two patients with severe liver involvement were started at a dose of 15 mg/m^2; in one patient the platelet count fell from 176,000 to 5000/mm^3 and the wbc count fell from 4400 to 200/mm^3, while the other patient had only mild thrombocytopenia. The wbc nadir was < 3000/mm^3 in 45% of the patients after the first course and the platelet nadir was < 75,000/mm^3 in 43%. Nadir counts were seen at a median of 22 days, with recovery 1–2 weeks later. There was no cumulative toxicity in the 20 patients who received multiple courses; ten patients received two courses, five received three courses, two received four courses, and one each received five, six, and nine courses. Gastrointestinal toxicity was mild to moderate and only two patients required a dose reduction in the subsequent course. Alopecia was observed in one patient. Extravasation led to local tissue necrosis in one patient.

COMMENT

One partial response (4%) was observed in the 26 patients who received dianhydrogalactitol. Since only patients with advanced disease were studied, it could be argued that this phase II trial was conducted under less than optimal conditions for detecting important clinical antitumor activity. However, under identical circumstances, the SWOG proved adriamycin to be an effective agent (3). From our results it is apparent that dianhydrogalactitol has a low degree of activity in breast cancer and further trials in breast cancer are not warranted.

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VP-16-213 Chemotherapy for Advanced Squamous Cell Carcinoma and Adenocarcinoma of the Lung [1,2]

Brief Reports and Preliminary Communications

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VP-16-213 is a semisynthetic podophyllotoxin derivative (1) which has been observed to have considerable activity against small cell undifferentiated lung cancer (2). Based on that encouraging clinical finding and the fact that the drug in the oral form has shown some activity against adenocarcinoma (ACA) of the lung as well (3), we elected to further evaluate the drug in patients with advanced squamous cell (SQC) and ACA of the lung.

MATERIALS AND METHODS

Forty-four patients with advanced SQC (20 patients) and ACA (24 patients) of the lung and no prior chemotherapy were entered in the study. Distant metastases were present in 21 of the 24 patients with ACA and in 15 of the 20 patients with SQC. Most patients without distant metastases were entered in this chemotherapy trial because their cardiopulmonary function precluded the use of surgery or radiation therapy. Patients with Eastern Cooperative Oncology Group (ECOG) performance scores of 4 were excluded from this study.

The pretreatment patient characteristics are shown in table 1. Indicator lesions were either measurable or evaluable. If measurable disease was present, standard definitions for complete regression (CR), partial regression (PR), stable disease, or progression were used (4). If evaluable but nonmeasurable disease was present, different definitions for response to therapy were used. A CR was the same as for patients with measurable disease. Other response categories were regression, stable disease, or progression and these categories required two observers to state unequivocally that there was either definite tumor shrinkage (regression), no appreciable change (stable disease), or definite tumor progression (progression). Our previous experience with this method of defining response to therapy has been published (4).

TABLE 1.—Pretreatment patient characteristics

	ACA	SQC
No. of patients	24	20
Median age in yrs	64	64
Range	42–74	42–82
Sex		
Men	16	20
Women	8	0
ECOG performance score		
0–1	13	9
2–3	11	11
Primary tumor resected	6	7
Primary tumor irradiated	3	2
Measurable indicator lesion	12	12
Evaluable indicator lesion	12	8

Treatment consisted of VP-16-213 given at a dose of 140 mg/m^2 as a 30-minute infusion on Days 1, 3, and 5 and repeated every 4–5 weeks from Day 1 of therapy until progression was noted. At progression, patients who met the original eligibility criteria and had neither cardiac nor renal disease were treated with the combination of cyclophosphamide (400 mg/m^2), adriamycin (40 mg/m^2), and *cis*-dichlorodiammineplatinum(II) (40 mg/m^2); all drugs were given by iv push. This latter regimen (known as CAP–I) has been reported previously (4). Doses for VP-16-213 were increased by 20% increments if no leukopenia or thrombocytopenia was noted and were decreased by 33% for wbc count nadirs of $< 2000/\text{mm}^3$ and/or platelet count nadirs of $< 75{,}000/\text{mm}^3$.

RESULTS

Among the 24 patients with ACA, 12 had measurable disease and 12 had evaluable disease. Among the 12 patients with measurable disease, two had PRs. Among the 12 patients with evaluable disease,

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one had a regression. Overall, three (12.5%) of 24 patients had tumor regressions. Twelve of the 20 patients with SQC had measurable disease and two of these 12 had PRs. Eight patients had evaluable disease and three of these eight had regressions. Overall, five (25%) of 20 patients had tumor regressions.

The mean time to tumor regression was 99 days (range, 31–168 days) for the three patients with ACA and 43 days (range, 24–69 days) for the five patients with SQC. Tumor regressions for the three ACA patients lasted 61, 105, and 133 days. Tumor regressions for the five SQC patients lasted 90, 133, 181, 187, and 218+ days. The duration of regression was calculated from the first day of tumor regression to the first day of tumor progression.

The median survivals were 173 days for all patients with ACA (range, 87–486+ days) and 219 days for all patients with SQC (range, 49–287+ days). The survivals were estimated from Kaplan-Meier survival plots. The three responders with ACA survived 245, 282, and 301+ days. The five responders with SQC survived 159, 209+, 212, 214+, and 232 days.

Among the 24 patients with ACA, 11 received subsequent treatment with CAP–I. Four (36%) of these 11 patients had tumor regressions (three PRs and one regression). Of the 20 patients with SQC, 12 received secondary treatment with CAP-I and two (17%) of these 12 responded (two PRs).

Toxic Effects

Toxicity from VP-16-213 was minimal and consisted primarily of mild-to-moderate leukopenia. Sixty-five percent of the patients with ACA and 70% of the patients with SQC developed leukopenia (wbc count $< 4000/\text{mm}^3$) during the course of their therapy. The median wbc count nadir was $3000/\text{mm}^3$ (range, $900–3800/\text{mm}^3$) for the patients with ACA developing leukopenia and $2800/\text{mm}^3$ (range, $700–3000/\text{mm}^3$) for the patients with SQC. No infections were noted. Only five patients developed thrombocytopenia (platelets $< 130{,}000/\text{mm}^3$) with the lowest platelet count being $80{,}000/\text{mm}^3$. Mild-to-moderate nausea, occasional vomiting, and moderate alopecia were the only other notable toxic effects.

DISCUSSION

VP-16-213 is a relatively well-tolerated chemotherapeutic agent when used in the manner described in this study. Definite antitumor activity was demonstrated in patients with both ACA and SQC of the lung. The overall regression rate of 18% (eight of 44 patients) when both ACA and SQC patients were combined, puts the drug in the same activity range as most single agents considered to have some effect in ACA and SQC of the lung such as cyclophosphamide (5,6) and adriamycin (7).

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LETTERS

Tumor Growth Kinetics, Therapeutic Differentials, and Design of Treatment Schedules

An Alternate View

As has been increasingly appreciated in recent years, most tumors follow Gompertzian rather than exponential growth kinetics (1–5) or, at least, usually grow more rapidly when small than when large. Since the characteristic slowing of the growth rate with increasing size is mainly due to a decreasing growth fraction, it has been proposed (6) that initial size reduction using a non-cycle-specific drug might, by expanding the growth fraction, render an increased proportion of tumor cells vulnerable to cycle-specific therapy. This proposal forms the basis for a clinical trial of such biphasic drug regimens in human multiple myeloma (7,8).

Very recently, Norton and Simon (9) presented, in a highly stimulating paper, a very general mathematic analysis of the response to cycle-specific therapy of cell populations following Gompertzian growth kinetics. This analysis shows maximum tumor sensitivity (ie, maximum regression rate) to occur at the inflexion point of the Gompertz curve. Norton and Simon (9) computed the effects of different types of treatment schedules, administering the same total amount of drug in the same total time, and observed that "late-intensity schedules" (the largest amounts of drug being given towards the end) produced greater cell kill than uniform or "early-intensity" schedules of more conventional design. These authors concluded that late-intensity schedules may therefore be clinically superior to other varieties. We believe this logic to be unsound and the conclusion reached to be unwarranted.

The fallacy consists in supposing that if schedule A is capable of effecting greater cell kill than schedule B, then the former is necessarily superior in clinical practice. However, this follows only if the two schedules are equally toxic to normal tissues (bone marrow, gastrointestinal tract, etc), or if schedule A is the less toxic of the two.

Increased tumor destruction can *always* be produced by simply raising the level of therapy, without regard to toxicity, but this is not clinically beneficial if the resultant toxicity is unacceptably dangerous. Such an effect can also be produced by changing the fractionation pattern, keeping total amounts of drug the same.

What is required, in order that clinical superiority may be inferred, is a demonstration that the recommended schedule leads to an improved *therapeutic differential* (ie, increased *ratio* of tumor to normal cell kill), not merely an increased tumor cell kill in terms of absolute numbers. Unfortunately, the analysis which led Norton and Simon to predict increased tumor cell kill by late-intensity schedules also leads to the identical conclusion for normal cell kill, but provides no indication of the direction in which the therapeutic differential will change. As Norton and Simon themselves noted, their analysis "is valid for any sigmoid ('S'-shaped) growth curve" whatever, and must surely hold for the repopulation curves of normal tissues as well as tumors. The clinical superiority of biphasic regimens utilizing early expansion of the growth fraction is likewise open to question, the growth fraction of (say) bone marrow stem cells being expanded along with that of the tumor—unless of course some time-scale difference in the two expansions is known and can be exploited.

These criticisms are not intended to detract from the thought-provoking qualities of the Norton-Simon analysis, further development of which is to be welcomed. Nor do they imply that late-intensity schedules and biphasic regimens must necessarily fail to display clinical superiority—an improved therapeutic differential being neither more nor less likely than its converse. Clinicians who undertake such trials should, however, be aware of the increased toxicity which is likely to result.

It is impossible in principle to predict superior treatment schedules from considerations of the tumor alone. Where, as in radiotherapy, attempts have been made to predict optimal schedules theoretically (10,11), such predictions are dependent on the existence of mathematic formulations which purport to compare the normal tissue damage inflicted by different treatment schedules (12–14). In chemotherapy, such formulations are still awaited. Consequently, improved treatment schedules cannot yet be derived theoretically, even in their most general form.

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Authors' Response

The argument raised by Drs. Wheldon and Brunton is less an objection to our model (1) than an expression of concern over the possible toxicity of clinical applications. We agree that considerations of normal tissue tolerance are important, but feel that their expectation of intolerable toxicity with a "late intensification" program may be overly pessimistic on several counts. From a clinical viewpoint many useful agents are felt to be more toxic to malignant tissues than to normal host tissues, especially over short periods of exposure (2). "Rescue" techniques (such as high-dose methotrexate with calcium leucovorin) may provide further dimensions for relatively non-toxic "intensive" therapies (3). Furthermore, advances in supportive care (antibiotics, blood product transfusions, and protected environments) may increase the "tolerability" of treatments in the future (4).

Of perhaps greater importance, however, is the role of dose scheduling in achieving an optimal therapeutic result. While the majority of tumors at presentation are well below their "plateau" size (as demonstrated by their continued growth if unsuccessfully treated), most normal tissues in the adult are close to their Gompertzian plateaus (that is, beyond point 6 in fig 2 of ref 1). At the initiation of therapy, therefore, the tumor is closer to the point of maximum growth rate and rate of regression than the normal tissues. The tumor may therefore be more affected by treatment, even if both normal and abnormal cells share identical biochemical response to therapy. Indeed, were it not for this discrepancy in growth rate, our ability to cause regression of even treatable tumors would perhaps be severely compromised. This is suggested by the difficulty experienced in treating even "sensitive" tumors like Hodgkin's lymphoma that are close to their plateau size at presentation.

Additionally, when tissue size is defined in terms of percent of maximum, many tumors have a slower specific growth rate than such normal tissues as bone marrow and gastrointestinal mucosa. That is, if C is the percent of maximum cancer tissue size and H is the percent of maximum size of a normal host tissue, if C' and H' are first derivatives with respect to time, and if K_1 and K_2 are constants, then

$$C'/C = K_1 \cdot 1n\,(100/C)$$
$$H'/H = K_2 \cdot 1n\,(100/H)$$
$$K_1 < K_2$$

This relationship is suggested by the common observation that a tumor may take months or years to grow from the in situ stage to an "advanced" stage, while many normal tissues can regrow to their "plateau" size in days or weeks following rather severe insults. Hence, tumor regrowth between cycles of therapy may lag considerably behind recovery of normal tissues, allowing for a greater overall therapeutic effect than toxic effect.

If cycles of intense therapy are required to cure small tumors, then these should be spaced as widely apart as is needed to provide for adequate host recovery. A reasonable approach to the design of post-surgical adjuvant chemotherapy, for example, would be to initiate treatment using low doses of active agents, with escalations in each subsequent cycle, treating as frequently as allowed by host recovery but postponing therapy between cycles if necessary to give the full planned dose. At the conclusion of such a protocol, short bursts of very intense treatment adequately spaced apart may be quite beneficial in causing regression of the small tumor, and may be well tolerated by host tissues close to their Gompertzian plateaus. In all, such a treatment plan may be considerably less toxic than some of those currently employed.

Lastly, it is important to emphasize that the model does not imply that "late intensification" *will* be successful in eradicating small tumor foci, but rather that the alternative concept (which advocates tapering of therapy as tumor size is reduced) is not the optimal plan of treatment. We may well discover that the maximum intensity achievable in the clinic with available agents is still insufficient for the cure of some small tumors. However disconcerting such a lesson, it would be important both practically and conceptually, and can only be learned from the analysis of well-designed clinical trials, not from verbal argument.

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March 27, 1978

Failure of Streptozotocin in Rare Hormonally Active Malignancies

Carcinomas arising from hormonally active tissues are rare. Also rare are malignancies which produce a detectable hormone in excess and a clinical syndrome as a result.

Streptozotocin, a naturally occurring nitrosourea-glucosamine antibiotic, has been shown to be active in producing both biochemical and tumor remissions in patients with metastatic islet cell carcinomas (1,2). Streptozotocin has been active in effecting changes not only in the secretion of insulin but also in the secretion of adrenocorticotropic hormone (ACTH) (3), parathormone-like substance (4,5), calcitonin (5), gastrin (6–8), glucagon (7,9), and serotonin (10) by islet cell carcinomas. Streptozotocin has also effectively improved the pancreatic cholera syndrome occurring with some islet cell carcinomas (11). Further, streptozotocin has been reported to be capable of producing remissions in a few cases of the serotonin-secreting malignant carcinoid tumor of the ileum (12,13). Other hormonally active tumors of amine precursor uptake and decarboxylation (APUD) origin may also respond to this agent.

At West Virginia University Hospital, two patients were recently seen who had rare hormone-secreting metastatic cancers. Both patients were treated with streptozotocin, and the results are reported here.

CASE REPORTS

Patient 1

A 61-year-old man was first seen because of a biopsy diagnosis of medullary carcinoma of the thyroid gland with confirmation of the tissue diagnosis at the Armed Forces Institute of Pathology. As part of a metastatic workup, a bone scan showed mild uptake in the spine. Bone marrow biopsies in the iliac crests revealed metastatic tumor in the marrow. A serum thyrocalcitonin level performed by immunoassay technique at the Mayo Clinic was 2.82 ng/ml (normal, < 0.155 ng/ml).

Adriamycin chemotherapy (60 mg/m^2 every 3 weeks) was initiated, and the patient received a total of three courses. A chest roentgenogram later showed new metastatic lesions whereas the previous film was normal. He did not respond to adriamycin. His treatment was switched to cyclophosphamide (1500 mg/m^2 every 3 weeks), and seven courses were given with stabilization of the metastatic tumor. Progression of tumor occurred and streptozotocin (1000 mg/m^2 weekly) was used next.

Six doses of this drug were administered (total dose, 11.4 g), and a chest roentgenogram showed tumor progression and more bone pain developed. The only toxic effect was severe vomiting; no renal toxic effects occurred. Serial determinations at weekly intervals of serum thyrocalcitonin were extraordinarily high and showed essentially no change (values were 10.16–12.5 ng/ml).

Patient 2

A 23-year-old man was seen because of metastatic thymoma. Ten months earlier he had developed symptoms and signs of florid, classic Cushing's syndrome, including moon facies, buffalo hump, abdominal stria, hypertension, and objective severe muscle weakness. Three years earlier, a mass was discovered on a chest roentgenogram and was surgically removed at another hospital. It was an epithelial thymoma. A current chest roentgenogram showed many parenchymal metastases bilaterally. A plasma cortisol was 63 μg/100 ml and there was loss of normal diurnal variation and no response to a high-dose dexamethasone suppression test. Ectopic production of ACTH by the thymoma was diagnosed.

Adriamycin (60 mg/m^2 on Day 1) and *cis*-dichlorodiammineplatinum(II) (20 mg/m^2 on Days 1–4) were administered for two courses at 4-week intervals. There was tumor progression, and a combination of cyclophosphamide (500 mg/m^2) and vincristine (1.4 mg/m^2) given on Days 1 and 8 with procarbazine (100 mg/m^2) given on Days 1–14 was begun (course repeated every 28 days). Stabilization of metastases observed on his chest roentgenogram occurred and lasted for 7.5 months. His lung metastases advanced and bone metastases appeared. Streptozotocin (1000 mg/m^2 weekly) was then used. Seven doses were given (total dose, 14.0 g) and the only toxic effect was vomiting. Subsequent chest roentgenograms showed tumor growth, and weekly plasma cortisols varied from 76 to 81 μg/100 ml (ie, no change).

Streptozotocin was used for each of these two rare hormonally active cancers because of the aforementioned reports of its effect in other similar APUD pancreatic carcinomas. Although both patients received an adequate trial of streptozotocin to produce a remission, neither one responded either biochemically or clinically by tumor measurement. The reason for this lack of response may be that streptozotocin is an active drug only in islet cell carcinomas. Negative results of streptozotocin use have been reported in two instances of malignant pheochromocytomas which are also APUD tumors (14). Perhaps a response would have occurred in these patients if streptozotocin had been the initial drug utilized.

Since not all reported cases of functioning islet cell carcinomas have responded to this drug (15), lack of drug activity in single case reports of other functioning cancers cannot be considered as a final result. However, both tumors in these two patients are very rare, and even more rarely metastatic. Thus, it is unlikely that anyone will encounter more than a sporadic case in need of chemotherapy. Others may wish to try streptozotocin as initial therapy in any such cancers observed; it would be important to report any remissions that occur.

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Unpredictable Hematologic Toxicity of Weekly DDMP

In the early 1950s, the compound DDMP [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine] was subjected to clinical trials. In daily doses of < 20 mg, DDMP caused rather severe hematologic toxicity, and no therapeutic benefit was observed among 22 patients with solid tumors (1).

Recently, renewed interest in DDMP has emerged with the administration of the drug in combination with citrovorum factor (CF) in an attempt to abolish the toxicity in hematopoietic tissue. In such studies, antitumor activity against various solid tumors has been demonstrated (2,3).

DDMP treatment with CF protection and rescue is presently under investigation, but the optimal dose schedule of DDMP and CF is unknown, and some delay of the CF rescue may be advisable (4). Accordingly, a phase I trial was initiated with the preliminary purpose of investigating the maximum tolerated dose of DDMP by weekly administration without CF rescue.

MATERIALS AND METHODS

All patients considered for this study had advanced malignant disease resistant to conventional chemotherapy and radiotherapy. Three patients had previously been treated with both irradiation and chemotherapy, one patient with irradiation alone, and four patients with chemotherapy alone.

The admission criteria included normal hematologic, liver, and renal status as evaluated from the pretreatment hemoglobin, wbc, and platelet counts, bone marrow examination, serum bilirubin, alkaline phosphatase, and creatinine. DDMP in plasma and folates in serum and erythrocytes were determined before treatment and before each subsequent administration of DDMP. (Analysis of folates included the total amount and was performed as a routine analysis by a ^{3}H-folate radioassay kit [CA-519].)

Initially, DDMP was given orally in a weekly dose of 25 mg/m^2 for 8 weeks. If no toxicity was noticed at this dose level, it was planned to increase the dose level according to a modified Fibonacci search scheme. Treatment with DDMP was withheld and CF rescue was given if hematologic toxic effects (wbc count $< 2500/\mu l$ and/or platelet count $< 100{,}000/\mu l$) occurred. The CF dosage depended on the grade of hematologic toxicity, varying from 25 mg/m^2 orally given once to 25 mg/m^2 iv given three times daily.

RESULTS

All nine patients were treated at the initial dose level of 25 mg/m^2 weekly. Pronounced toxic effects (wbc count $< 1000/\mu l$ and/or platelet count $< 30{,}000/\mu l$) occurred in five patients. Thrombocytopenia alone was seen in three of these patients, and thrombocytopenia together with leukopenia were seen in two patients. The latter two patients died from drug-related toxic effects, one from cerebral bleeding and one from infection.

In two of the remaining four patients, no or only mild toxic effects with platelet counts of 75,000–100,000/μl developed.

Pronounced hematologic toxic effects occurred after two or three courses in the five patients. The two patients with mild thrombocytopenia had three and six courses respectively, while two patients received the planned treatment for > 8 weeks without hematologic toxicity and thus without the need for CF.

Hemoglobin levels and wbc and platelet counts were followed twice weekly and CF rescue, in a dose according to the grade of toxicity, was given at the first sign of hematologic toxicity. As noted, two patients died from toxic reactions while the remaining patients were rescued within 1 week without complications.

The pretreatment level of folates in serum and erythrocytes was significantly lower in the group with pronounced toxic effects than in the group with no or mild toxic effects ($P < 0.05$, Mann-Whitney). Patients with higher folate levels seemed to tolerate a higher total dose of DDMP without toxic effects. No significant difference was found between the plasma levels of DDMP in the two groups, the highest determined value being 0.29 μg/ml in the group with pronounced toxicity and 0.41 μg/ml in the other group. The median ages in the two groups were almost identical (57 and 58 years).

DISCUSSION

In this study, life-threatening toxic effects occurred in some patients within 4 weeks at *weekly* doses of from 37.5 to 50 mg, while other patients tolerated the same regimen without signs of toxicity. This indicates a rather unpredictable hematologic toxicity from treatment with DDMP similar to that observed with methotrexate treatment (5).

It is noteworthy that hematologic toxic effects were confined particularly to thrombocytopenia, while leukopenia never occurred alone. It is also of clinical interest that the pretreatment level of folates may be a predictive factor for the toxic property of the drug. Although the difference found in this trial may be a reflection of the performance status, similar observations were made by Miller et al (2).

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A Response to the Guidelines for Training in Medical Oncology

The recently published (1) Guidelines for Training in Medical Oncology were a source of great disappointment and concern to me. There were several pages of detailed recommendations about education regarding cell kinetics, biochemistry, pharmacology, etc (all, of course, being important), but there was only one brief line about "psychosocial areas."

As a practicing medical oncologist, at least 80% of my energy is spent as a family therapist and psychologic counselor. Such areas are rarely, if ever, adequately covered in fellowship training programs, and consequently I am one of a few psychiatrists in this country who understand the S phase of the cell cycle but no family theory. Inasmuch as chemotherapy for the common cancers is of little or no real value, I feel it is imperative that we do not allow young physicians to become oncologists without being aware of this fact and without receiving a great deal of training in the management of emotional problems. Only if they enter the field with no illusions about what it really is can they appropriately prepare themselves.

There needs to be a major re-appraisal and refocus of our oncologic training programs, or there is a grave risk that we will produce oncologists who either absorb themselves in issues of false importance (eg, giving elaborate protocols that have little real benefit) or become disillusioned altogether and leave the field. I believe medical oncology as a discipline does indeed have much to offer cancer patients and their families, but only if it realistically focuses on its capabilities.

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April 17, 1978

ANNOUNCEMENTS

Symposium on "New Drugs in Cancer Therapy"

Institut Jules Bordet, Brussels, Belgium, September 7–8, 1978

The National Cancer Institute (NCI) and the European Organization for Research on Treatment of Cancer (EORTC) announce a symposium on "New Drugs in Cancer Therapy" to be held September 7 (EORTC and NCI Investigational Drugs) and 8 (Adriamycin and Anthracycline Derivatives), 1978, at 2, rue Evers (1st floor), Institut Jules Bordet, 1000 Brussels, Belgium.

The number of participants will be limited to 200. The Chairmen are Drs. F. M. Muggia and H. J. Tagnon. Among the participants are: P. Alberto (Geneva, Switzerland), N. Bachur (Baltimore, Md), L. Baker (Detroit, Mich), R. Benjamin (Houston, Tex), G. Bonadonna, M. Casazza, S. Garattini, M. Ghione, and C. Praga (Milan, Italy), S. K. Carter (Palo Alto, Calif), H. Cortes-Funes (Madrid, Spain), J. Daniels (Stanford, Calif), H. Davis (Madison, Wis), A. Goldin, F. Muggia, C. Myers, M. Rozencweig, and D. Von Hoff (Bethesda, Md), H. Hansen (Copenhagen, Denmark), D. Henry (Research Triangle Park, NC), Y. Kenis, M. Staquet, H. Tagnon, and A. Trouet (Brussels, Belgium), and G. Mathé (Paris, France).

The registration fee is 3000 Belgian francs. A small number of free communications will be integrated in the program. Abstracts must be sent to Dr. M. Rozencweig, Division of Cancer Treatment, NCI, Bldg 37, Rm 6A17, Bethesda, Md 20014, *before* July 15, 1978. *For details and registration write to:* Dr. M. Staquet, EORTC Data Center, Institut Jules Bordet, rue Héger-Bordet 1, 1000 Brussels, Belgium (Phone: 538.57.90-Telex: 22773).

Fourth Congress of the Medical Oncology Society

Nice, France, December 2–4, 1978

The Medical Oncology Society, Nice, France, announces the "Fourth Congress of the Medical Oncology Society" to be held December 2–4, 1978.

Direct inquiries to: Dr. M. Schneider, Centre Antoine Lacassagne, 36 Voie Romaine, 06054 Nice, Cedex, France (93) 53.10.10.

Fellowship in Medical Oncology

Applications are being accepted from candidates who are interested in a Joint Collaborative Training Program in Medical Oncology involving major teaching hospitals of Brown University's Program in Medicine, beginning July 1979 and July 1980. This program offers clinical and research activities in clinical pharmacology and cancer chemotherapy, biochemical pharmacology, drug development, and tumor immunology, and participates in national cooperative chemotherapy groups to aid in the management of patients with solid tumors and hematologic malignancies seen at many of the hospitals in the Providence, Rhode Island, area.

Prerequisites include 2 years of approved Medical Residency and American Board of Internal Medicine eligibility. Candidates with academic interests are encouraged to apply. *Send letter and curriculum vitae to:* Paul Calabresi, MD, Professor and Chairman, Department of Medicine, or F. J. Cummings, MD, Head, Division of Medical Oncology, Department of Medicine, Roger Williams General Hospital, 825 Chalkstone Ave, Providence, RI 02908.

Seminars in Oncology at Roswell Park Memorial Institute

Buffalo, New York

There will be a seminar on "General Oncology" to be held in July–August 1978, organized by the Department of Pathophysiology at Roswell Park Memorial Institute. The chairperson is Dr. Julian Ambrus.

There will be a $10.00 registration fee. *For further information contact:* Ms. Claudia Lee, Cancer Control, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

23rd Annual Clinical Conference on "Cancer of the Genitourinary Tract"

Houston, Texas, November 2–3, 1978

The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute in Houston, announces the 1978 Clinical Conference on "Cancer of the Genitourinary Tract" to be held at the Shamrock Hilton Hotel, Houston, Texas, November 2–3, 1978. This Conference will focus on issues of diagnosis, staging, and therapy as they relate to the critical and sometimes controversial therapies of the multiple stages of urologic malignancies. The Conference will be jointly sponsored by The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute and the American Cancer Society, Texas Division, Inc. It will provide practicing primary care physicians, oncologists, and urologists with critical issues at the moving edge of therapeutic investigation on urologic malignancies. An opportunity will be provided for speakers and registrants to exchange information at the conclusion of each session. There will be a registration fee.

This medical-education offering meets the criteria for 14 hours in Category I of the Physician's Recognition Award of the American Medical Association. This program is also acceptable for 14 prescribed hours by the American Academy of Family Physicians.

For additional information write: George R. Blumenschein, M. D., Office of Education, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Tex 77030.

Monograph on Non-Hodgkin's Lymphoma

A limited number of copies of the collected papers from the National Cancer Institute-CCIRC symposium on Non-Hodgkin's lymphomas which was held in San Francisco, Calif, in 1976 are available without charge. *Please contact:* Stephen E. Jones, MD, Section of Hematology-Medical Oncology, University of Arizona Cancer Center, Arizona Health Sciences Center, Tucson, Ariz 85724, in writing for a copy.

Conference on "Combined Modalities: Chemotherapy/Radiotherapy"

Hilton Head, South Carolina, November 15–18, 1978

The Committee for Radiation Oncology Studies, in conjunction with members of the Radiation Study Section and leading oncologists and with the support of the National Cancer Institute, is sponsoring a conference on the interaction of chemotherapy and radiotherapy. The meeting will focus on experimental results of laboratory and clinical studies of the interaction of selected cancer chemotherapeutic agents and radiation. The emphasis will be on the response of both normal and tumor tissues.

Attendance will be limited to people working in the field, certain invited speakers and participants, and those investigators who submit abstracts that are accepted by the organizing committee. It is intended to be a workshop-type meeting and abstracts will be presented either in open session or as poster-scientific exhibit sessions.

Data to be presented at the meeting should meet the following criteria: (*a*) data should not have been published previously; (*b*) data must be relevant to the subjects of the meeting listed below and (*c*) a paper must be prepared and submitted for publication at the time of the meeting, whether to be presented as an exhibit or oral presentation. The deadline for submission of abstracts will be August 1, 1978. Persons desiring to attend, whose abstracts are accepted, will be eligible for partial travel support to the meeting. Additional details as to the format of the papers will be sent to those proffering accepted abstracts.

The research to be reported will include pharmacology, biochemistry, and basic chemical effects of the specific compounds, in vitro results with interaction of drug and radiation, in vivo normal tissue response to drug and radiation, and current clinical trial information. The following drugs are of special interest: bleomycin, adriamycin, cyclophosphamide, *cis*-platinum, and nitrosoureas. Papers on other agents will be considered for a session on other interesting and new agents. In addition to inviting the submission of abstracts, the organizing committee also invites nominations of young investigators who will be supported for travel to the meeting and who will not necessarily be presenting a paper.

For further details on the meeting and for abstract forms, write to: Theodore L. Phillips, MD, Chairman, Organizing Committee, Division of Radiation Oncology, University of California, M-330, San Francisco, Calif 94143.

Fifth Annual Chicago Symposium on "Cancer Immunology: Experimental and Clinical"

Chicago, Illinois, September 13–15, 1978

The Fifth Annual Chicago Symposium entitled "Cancer Immunology: Experimental and Clinical" is sponsored by the ITR-Biomedical Research and the University of Illinois at the Medical Center, the Illinois Cancer Council, and the American Cancer Society. It will be held September 13–15, 1978, at the Pick-Congress Hotel, 520 S Michigan Ave, Chicago, Ill.

It is the purpose of this symposium to provide an up-to-date review as well as information on recent developments in immunodiagnosis, immune response, and the immunologic aspects of therapy. Papers by invited guest speakers will be presented in the following four areas of concentration: Cancer Immunodiagnosis and Viral Immunity (*Chairman: Saul Kitt*, Division of Biochemical Virology, Baylor College of Medicine); Tumor Antigens and Modulators of Immunity (*Chairman: Isaiah J. Fidler*, Head, Biology of Metastasis, Frederick Cancer Research Center); Immunologic Aspects of Therapy (*Chairman: Evan Hersh*, Chief, Section on Immunology, M. D. Anderson Hospital and Tumor Institute); and Cellular and Humoral Immunity (*Chairman: Karl Hellstrom*, Division of Tumor Immunology, Fred Hutchinson Cancer Research Center).

Attendance is open to all interested medical investigators and physicians. The registration fee is $70.00 and includes abstracts, coffee, refreshments, and luncheon and evening dinner banquet on Thursday. We are honored to have Dr. George Mathé as our speaker on that occasion.

For further information, please contact: Dr. Ray G. Crispen, Convener, ITR-Biomedical Research, 904 W Adams St, Chicago, Ill 60607.

Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics and the Society of Toxicology

Houston, Texas, August 13–17, 1978

The 1978 Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics and the Society of Toxicology will be held at the University of Houston in Texas.

In addition to a diversity of scientific sessions, the meeting will also feature symposia on the following topics: Transmembrane Passage of Substances, Quantification of Drugs in Biological Fluids, Drug Metabolism and the Safety of Xenobiotics, Tachyphylaxis to Catecholamines, Cancer Chemotherapy, and the Role of Epidemiology in Toxicology.

For information and registration contact: ASPET-SOT, College of Pharmacy 141 SR-2, University of Houston, Houston, Tex 77004.

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